

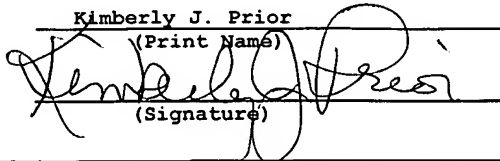


CERTIFICATE OF MAILING (37 CFR 1.8a)

1FW

I hereby certify that this paper (along with any paper referred to as being transmitted therewith) is being deposited with the United States Postal Service on the date shown below with sufficient postage as first class mail in an envelope addressed to the: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Date: March 30, 2005

Kimberly J. Prior
(Print Name)

(Signature)

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Group No.: 1645

Ulrich Certa, et al.

Serial No.: 10/735,973

Filed: December 15, 2003

For: **MODIFIED PHOSPHODIESTERASE POLYPEPTIDES WITH ALTERED PHYSIOCHEMICAL PROPERTIES**

TRANSMITTAL OF CERTIFIED COPY

March 30, 2005

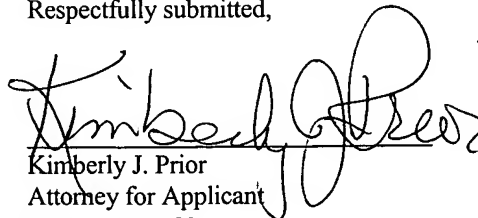
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Attached please find the certified copy of the foreign application from which priority is claimed for this case:

<u>Country</u>	<u>Application No.</u>	<u>Filing Date</u>
Europe	02028057.4	December 17, 2002

Respectfully submitted,



Kimberly J. Prior
Attorney for Applicant
Reg. No. 41,483
Hoffmann-La Roche Inc.
340 Kingsland Street
Nutley, New Jersey 07110
Phone: (973) 235-6208

KJP/bah
Enclosures

This Page Blank (uspto)



**Europäisches
Patentamt**

**European
Patent Office**

**Office européen
des brevets**

Bescheinigung

Certificate

Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

02028057.4

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

This Page Blank (uspto)



Anmeldung Nr:
Application no.: 02028057.4
Demande no:

Anmeldetag:
Date of filing: 17.12.02
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

F. HOFFMANN-LA ROCHE AG

4070 Basel
SUISSE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se référer à la description.)

Modified phosphodiesterase polypeptides with altered physicochemical properties

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s)
revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C07K14/00

Anmeldetag benannte Vertragsstaaten/Contracting states designated at date of
filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR IE IT LI LU MC NL PT SE SI SK

Page Blank (16pt)

Modified phosphodiesterase polypeptides with altered physicochemical properties

The invention relates to modified phosphodiesterase (PDE) polypeptides with altered physicochemical properties which are particularly useful in screening assays for the
5 identification of agonists or antagonists of PDEs, the polynucleotides encoding such modified PDE polypeptides, antibodies against such polypeptides, methods of preparation and uses of such polypeptides.

The phosphodiesterases (PDEs) represent a family of enzymes that catalyze the
10 hydrolysis of the various cyclic nucleoside monophosphates (including cAMP). These cyclic nucleotides have been found to act as second messengers within the cells by carrying impulses from cell surface receptors having bound various hormones and neurotransmitters to the inside of the cells. The task of phosphodiesterases is to degrade these cyclic mononucleotides once their messenger role is completed thereby regulating
15 the level of cyclic nucleotides within the cells and maintaining cyclic nucleotide homeostasis.

Ten families of PDEs have been identified, designated PDE1 to PDE10. Within each family there are two or more related but distinct gene products (A, B, C, etc.) and for each
20 of these alternative mRNA processing gives rise to multiple splice variants, identified by an additional arabic numeral in accordance with the most recent nomenclature recommendation (Molecular Pharmacology 46, 399-405, 1994).

All PDE gene products identified so far have one catalytic and a least one regulatory domain. The catalytic domain lies towards the carboxylic acid terminus of each PDE protein and has the greatest homology between the PDE families, being >75% homologous at the amino acid level (Perry and Higgs, *Curr. Opin. Chem. Biol.* 2, 472-481, 1998). Nevertheless, each of the more than 30 known PDEs have individually distinct substrate specificities, kinetic characteristics, regulatory properties and cellular and subcellular distributions (Houslay and Milligan, *Trends in Biochem. Sci.* 22, 217-224, 1997).

PDEs 4, 7 and 8 are highly specific for cAMP. PDEs 5, 6, 9 and 10 are selective for cGMP. PDE3s bind cAMP and cGMP with similar affinity, but hydrolyse cAMP most efficiently, cGMP rather poorly. PDE3s are, therefore, negatively regulated in their cAMP hydrolysing ability by cGMP. PDEs 1 and 2 hydrolyse both cAMP and cGMP, but with PDE1 the relative efficiencies vary with isoenzyme subtype (Perry and Higgs, *Curr. Opin. Chem. Biol.* 2, 472-481, 1998).

The amino terminal ends of PDEs consist of the regulatory domains, which are very different both between families and between variants within families. This region contains variously: a binding domain for Ca²⁺-calmodulin (CaM) in PDE1 ; non-catalytic cGMP-35 binding sites in PDEs 2, 5 and 6; a binding domain for the signalling G-protein transducin in PDE6. The amino terminal region also contains protein- and membrane-targeting sequences in several PDE3s and PDE4s, as well as protein kinase phosphorylation sites in PDEs 1, 3, 4 and 5. These phosphorylation sites are likely to be important in regulation of catalytic activity and/or subcellular location (Perry and Higgs, *Curr. Opin. Chem. Biol.* 2, 472-481, 1998).

Of particular interest to this invention are the PDE4 enzymes. PDE4 enzymes share a common structure, as deduced from their amino acid sequences (Beavo and Reifsnnyder, *Trends Pharmacol. Sci.* 11, 150-155, 1990; Bolger et al., *Mol. Cell Biol.* 13, 6558-6571, 1993; Houslay, Sullivan and Bolger, *Adv. Pharmacol.* 44, 225-242, 1998). Members of each gene family (PDE4A, PDE4B, PDE4C, PDE4D) share common C-terminal regions, different for each family, and catalytic domains that for all PDE4 isoforms are very similar

(84% homology over about 360 amino acids across all PDE4s; Houslay, Sullivan and Bolger, *Adv. Pharmacol.* 44, 225-242, 1998).

From N-terminus to catalytic region, the sequence in "long form" PDE4s can be
5 divided into 5 regions, three of which are isoform-specific (N-terminal region, linker
regions 1 and 2, or LR1 and LR2) and two, more conserved regions, that are broadly
similar between all isoforms, the upstream conserved regions 1 and 2 (UCR1 and UCR2).
"Short form" PDE4s, e.g. PDE4A1, PDE4B2, PDE4D1, PDE4D2, lack UCR1 and LR1 plus
differing amounts of the N-terminal region of UCR2. Throughout all regions are potential
10 phosphorylation sites for a variety of kinases, including PKA (e.g. Ser 54 in human
PDE4D3), mitogen activated protein kinases (e.g. Ser 487 of human PDE4B2), casein
kinase II (e.g. Ser 489 of PDE4B2) and calcium-diacylglycerol dependent protein kinases
(Houslay, Sullivan and Bolger, *Adv. Pharmacol.* 44, 225-242, 1998). Phosphorylations at
some of these sites have been shown to activate the PDEs (e.g. Ser 54), others serve to
15 inhibit. There is also evidence that some phosphorylations serve to prime the enzymes
ready for subsequent activation by further phosphorylation at a different site or sites
(Houslay, Sullivan and Bolger, *Adv. Pharmacol.* 44, 225-242, 1998). Other auto-regulatory
sites may be found in the N-terminal sequence of certain PDE4s (Bolger et al., *Mol. Cell*
Biol. 13, 6558-6571, 1993, Mc Phee et al., *Biochem. J.* 310, 965-974, 1995).

20

Specific PDE4 inhibition may be useful over a very wide range of disease areas. These
include: asthma, atopic dermatitis, depression, reperfusion injury, septic shock, toxic
shock, autoimmune diabetes, AIDS, Crohn's disease, multiple sclerosis, cerebral ischemia,
psoriasis, allograft rejection, restenosis, stroke, ulcerative colitis, cachexia, cerebral malaria,
25 allergic rhinoconjunctivitis, osteoarthritis, rheumatoid arthritis, autoimmune
encephalomyelitis (Houslay, Sullivan and Bolger, *Adv. Pharmacol.* 44, 225-242, 1998,
WO02/074992). In the area of asthma, PDE4 inhibition helps to increase cAMP in
bronchial smooth muscle, thereby producing a modest bronchodilatory effect, of use in
the alleviation of asthmatic symptoms. But perhaps most importantly, inhibition of PDE4s
30 is now a recognised method by which to suppress immune and inflammatory cell
responses (Hughes et al. *Drug Discov. Today* 2, 89-101, 1997; Torphy, *Am. J. Respir. Crit.*
Care Med. 157, 351-370, 1998; Teixeira et al., *Trends Pharm. Sci.* 18, 164-171, 1997).

PDE4s play major roles in modulating the activity of virtually every cell type involved in the inflammatory process. Immune and inflammatory conditions occur when recruitment of leukocytes from the blood compartment into tissues is either uncontrolled, inappropriate, prolonged or directed against self. In asthma, rheumatoid arthritis and multiple sclerosis, infiltration of tissues with inflammatory cells is prolonged and intense, leading ultimately to severe (and self-perpetuating) damage and loss of function. Acute dysregulation of the immune system occurs in such conditions as acute respiratory distress syndrome (ARDS) where an overwhelming and generalised inflammatory response can frequently lead to death. There is also substantial evidence which suggests that inflammation may play a part in defining the extent of injury resulting from reperfusion following ischaemia, at least in brain and lung (Entman and Smith, *Cardiovasc. Res.* 28, 1301-1311, 1994).

The PDE4D phosphodiesterases, which are the most preferred focus of the present invention, exist in mammals in the form of isoenzymes (which represent different molecular forms of the same enzyme polypeptide). These phosphodiesterases are localized in the cytosol of the cell and are unassociated with any known membraneous structures. The PDE4D isoenzymes specifically degrade cAMP and are a common target for such pharmacological agents as antidepressants (for example, rolipram). Several splice forms of PDE4D are known. the short forms, PDE4D1 and PDE4D2, lack UCR1 and LR1 sites. Rather than these, interest of the present invention focuses on the long isoforms, of which 6 are known, namely PDE4D3, PDE4D4, PDE4D5, PDE4D6, PDE4D7 and PDE4D8. All of these have in common the LR1 and UCR1 sites and the domains located at the C-terminus of these sites, but they have different N-terminal domains. Isoforms PDE4D6, D7 and D8 were recently disclosed in WO02/074992.

For identification of compounds that inhibit PDE4D isoenzymes, screening assays are used that are either whole cell assays in which the cells are treated with the inhibitor and cAMP hydrolysis is measured, or in vitro assays which use purified PDE4D isoenzymes either isolated from their natural sources or from cells overexpressing recombinant PDE4D isoenzymes. Such assays are described e.g. in WO00/09504, WO01/35979, WO01/68600 and WO98/45268. WO01/35979 describes the purification of recombinant PDE4D expressed in *Saccharomyces Cerevisiae*. The use of recombinant native forms of PDE4D for the identification of PDE agonists or antagonists has the

disadvantage that the PDE4D polypeptide does not have optimal physicochemical characteristics, especially with respect to aggregate formation. Aggregate formation can lead to protein precipitation and is known to potentially decrease the activity of enzymes, compared to the authentic oligomeric molecules. Thus, aggregation of the native forms of PDE4D polypeptides in screening assays aimed at identifying agonists or antagonists of PDE4D phosphodiesterase activity negatively affects the quality of these assays. In addition, the use of assays based on individual recombinant PDE4D isoforms has the disadvantage of potentially resulting in the identification of agonists or antagonists that are specific for a single isoform of a PDE4D only, instead of agonists or antagonists which act on all long or short form PDE4D isoforms together.

Figure 1: Specific phosphodiesterase activity for the different PDE4D isoforms D3 and D5 to D8, and the PDE4D core construct (DC).

Figure 2: Aggregate formation by different PDE4D isoforms and the core construct. In this figure, the gel filtration profiles of different isoforms of PDE4D (D3, D5, D6, D7, D8), and the core construct (DC) are shown. The first elution peak represents PDE4D aggregates. The pronounced shoulder in the elution profile of DC, where polypeptides of approx. 300,000 Dalton elute, indicates a decrease in aggregation of DC, as compared to the five isoforms of PDE4D.

Figure 3: Quantitation of aggregation of the core construct (DC) of PDE4D, and of the tubulin content of the core construct preparations. In A), the elution profile of DC is shown, with the fractions indicated on the bottom. B) shows an SDS-PAGE of the different fractions from the gel filtration. C) shows the analysis of optical densities of individual fractions corresponding to the Coomassie stained SDS-PAGE in B).

Figure 4: Differences in aggregation and tubulin content of different isoforms and the core construct of PDE. A) SDS-PAGE gels are shown for DC and five isoforms of PDE4D which were analysed as described above. B) The percentage of non-aggregated PDE4D is shown for the core construct and the isoforms D3 and D5 to D8, based on total PDE4D protein. DC has the highest percentage of non-aggregated PDE4D, as compared to

the five isoforms. C) This figure shows the ratio between PDE4D and tubulin for DC and the isoforms D3 and D5 to D8, based on total PDE mass and total tubulin mass. As can be seen, the tubulin content of DC is significantly lower than that of the five PDE4D isoforms indicated.

5

Figure 5: Activity assay. Phosphodiesterase activity is shown both for the PDE4D core construct (DC) and PDE4D3 (D3).

Figure 6: Rolipram inhibition. Phosphodiesterase activity of the PDE4D core
10 construct (DC) can be inhibited by Rolipram. The IC₅₀ of DC phosphodiesterase activity by Rolipram inhibition was 0.34+/- 0.06 mM.

The term "polypeptide" as used herein, refers to a polymer of amino acids, and not to
15 a specific length. Thus, peptides, oligopeptides and proteins are included within the definition of polypeptide.

The term "physicochemical properties" as used herein refers to the propensity of polypeptides to monomer formation, oligomer formation or aggregate formation.

20

The terms "aggregation" and "aggregate formation" as used herein are used interchangeably and refer to the type of association of polypeptide chains which, in contrast to oligomer formation, is an irreversible process and does not follow the law of mass interaction. Aggregation progresses in a time dependent manner and does not stop
25 when a defined number of chains have associated. Aggregation of polypeptides can lead to protein precipitation because the molecular size of the aggregate is so dramatically increased that the solubility of the molecules is impaired. Aggregation is also known to potentially decrease the activity of the enzyme as compared to authentic oligomeric molecules.

The term "oligomer formation" as used herein refers to a reversible association of polypeptide chains whereby the ratio of oligomeric proteins and monomeric proteins at a certain protein concentration and at thermodynamic equilibrium is described by the
5 association constant.

The term "non-aggregated" refers to polypeptides that are present either as oligomers or monomers or a mixture of oligomers and monomers at equilibrium according to the law of mass interaction.

10

The term "improved physicochemical properties" as used herein refers to a decrease in aggregate formation of polypeptides. For the purpose of definition, aggregate formation of the polypeptides of this invention is determined by quantitation of the fractions of a gelfiltration experiment in which aggregated PDE elutes, and of the fractions in which
15 non-aggregated PDE elutes. The degree of aggregation is calculated in percent of non-aggregated PDE based on total (aggregated and non-aggregated) PDE. For the purpose of the definition of aggregation, aggregated PDE is considered to elute in fractions 2 to 4, in which aggregated molecules of at least 2 Mio Daltons elute, and non-aggregated PDE is considered to elute in fractions 5 to 11, corresponding to the fractions in which molecules
20 of about 0.3 Mio Daltons elute, when the following conditions are used for gel filtration: 50 µl of Ni-NTA agarose purified isoform preparation are injected into a Superose 12 size exclusion column (type PC3.2/30; Amersham Pharmacia Biotech), equilibrated with 50 mM TrisHCl pH 7.7, 100 mM NaCl, 0.5 mM MgCl₂ at a flow rate of 0.1 ml/min at 4°C. Chromatograms are recorded at 278 nm. Starting from the elution volume onwards, the
25 column eluate is collected as 50 µl fractions.

The term "long form PDE" as used herein refers to PDE isoforms which comprise an isoform specific N-terminal domain, linker regions LR1 and/or LR2, and upstream conserved regions UCR1 and UCR2.

30

The term "UCR1 start" as used herein refers to the first amino acid of the UCR1 domain of PDE4D, as annotated in SEQ ID No. 1 to 7.

5 The term "LF1 site" as used herein refers to the first amino acid of the splice site of PDE4D as indicated in SEQ ID No. 1-7.

The term "amino acids upstream of LF1" refers to the position of an amino acid counted from the LF-1 site towards the amino terminus of the native protein.

10 The term "D3" as used herein refers to the PDE4D3 isoform. The term "D4" as used herein refers to the PDE4D4 isoform. The term "D5" as used herein refers to the PDE4D5 isoform. The term "D6" as used herein refers to the PDE4D6 isoform as provided by WO02/074992. The term "D7" as used herein refers to the PDE4D7 isoform as provided by WO02/074992. The term "D8" as used herein refers to the PDE4D8 isoform as provided by
15 WO02/074992. The polypeptide sequences of the human PDE4D isoforms D3, D4, D5, D6, D7, and D8 are shown in SEQ ID No.1 and 3-7.

The term "Ser54" as used herein refers to the Ser residue in position 54 of PDE4D3, and to the Ser residue in the respective position of any of the other PDE polypeptides. The
20 position corresponding to Ser54 of PDE4D3 is annotated in SEQ ID No. 1 to 7 for the different isoforms and the core sequence which is common to all the PDE4D isoforms.

The term "Ser579" as used herein refers to the Ser residue in position 579 of PDE4D3, and to the Ser residue in the respective position of any of the other PDE
25 polypeptides. The position corresponding to Ser579 of PDE4D3 is annotated in SEQ ID No. 1 to 7 for the different isoforms and the core sequence which is common to all the PDE4D isoforms.

The term "tubulin association" as used herein refers to the tubulin content of purified PDE preparations.

As used herein, the term "agonist" also refers to activators, and the term "antagonist" also refers to inhibitors of PDE phosphodiesterase activity.

The disadvantage of native recombinant PDE polypeptides resides in the formation of aggregates of the polypeptides, which gives rise to sub-optimal conditions for the identification of modulators of PDE phosphodiesterase activity in screening assays since aggregate formation is known to lead to polypeptide precipitation and to potentially impair activity of a polypeptide. The present invention is directed to the generation of PDE polypeptides with improved physicochemical properties which are particularly useful for the purpose of identifying agonists or antagonists of these enzymes.

15

The problem of aggregate formation of recombinant PDE polypeptides was overcome in the present invention by generating a polypeptide comprising a PDE polypeptide sequence with an amino-terminal deletion, said polypeptide exhibiting decreased aggregate formation, as shown in Figures 2 and 3. These polypeptides have a specific activity that is comparable to the specific activity of the native recombinant proteins, as is shown in Figure 1. In a preferred embodiment, the invention provides a polypeptide comprising a PDE polypeptide sequence with an amino-terminal deletion wherein the proportion of non-aggregated PDE in the total protein preparation is from 55 %, preferably from 60%, more preferably from 65%, most preferably from 68% to 100%, preferably to 90 %, more preferably to 80 %, most preferably to 70% of total protein, as determined by the quantitation of eluted fractions from a gel filtration of the PDE fractions. Quantitation is performed as follows: Equal volume aliquots of fractions from Size Exclusion Chromatography, run as hereinbefore described, are analyzed by SDS-PAGE (one gel per run and isoform, Figure 3). Optical densities of Coomassie stained PDE are integrated as follows: After electrophoresis the Coomassie stained polyacrylamide gel was imaged by a video imaging system (Figure 1). Optical densities of PDE bands were integrated using a Macintosh computer and the public domain software "NIH Image",

version 1.61 (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). The integrated arbitrary units per PDE band as returned by the software reflect the relative PDE concentrations within the original pools. Integrated densities from PDE bands of fractions 2-4 are added up, giving a
5 relative amount of aggregated PDE, PDE_{agg} (Figure 3). These fractions contain molecules of at least 2 Mio Daltons. Similarly, integrated densities from PDE bands of fractions 5-11 are added up, giving a relative amount of non-aggregated PDE, $PDE_{non-agg}$. In fractions 5-11, molecules in the range of 0.3 Mio Daltons elute. Relative amount of total PDE, PDE_{total} , is given by the sum of PDE_{agg} and $PDE_{non-agg}$. The percentage of non-aggregated PDE is
10 given by the ratio of $PDE_{non-agg}$ and PDE_{total} , multiplied by 100.

In a preferred embodiment of the present invention the PDE polypeptide sequence with an amino-terminal deletion is a long form PDE polypeptide sequence. In a more preferred embodiment, the PDE polypeptide sequence is a PDE4 polypeptide sequence,
15 preferably a long form PDE4 polypeptide sequence. In a most preferred embodiment, the polypeptide sequence is a PDE4D polypeptide sequence, preferably a long form PDE4D polypeptide sequence.

Methods of generating amino-terminal deletion mutants of polypeptides are well
20 known. Such deletion mutants can be generated by using site directed mutagenesis, e.g. by using PCR (Sambrook et al., Molecular Cloning: A Laboratory Manual (1989), Cold Spring Harbor Laboratory Press, New York, USA).

In one embodiment of the present invention, the polypeptide comprises a PDE
25 polypeptide sequence starting between 25 amino acids, preferably between 20 amino acids, more preferably between 15 amino acids, even more preferably between 10 amino acids, most preferably between 5 amino acids upstream of LF1 and the LF1 site.

In another embodiment the PDE polypeptide sequence starts at any amino acid
30 located between the LF1 splice site and the first amino acid of the UCR1 start of the native PDE polypeptide. In the case of the most preferred embodiment of the PDE polypeptide, PDE4D, these sites are identical in all of the long form PDE4D isoforms.

In another more preferred embodiment of the present invention, the amino-terminal deletion is such that the PDE polypeptide sequence starts at the LF1 splice site of the native PDE polypeptide. This PDE polypeptide sequence is referred to as the "core construct". The core construct spans a sequence of PDE which is shared by all isoforms.
5 The core construct sequence of PDE4D is shown in SEQ ID No. 2.

In another more preferred embodiment of the present invention, the PDE polypeptide sequence start is located 13 amino acids upstream of the UCR1 start of the
10 native PDE polypeptide. The location of this position in the long form PDE4D isoforms and the core construct of PDE4D is shown in SEQ ID No. 1-7.

The basis for the generation of the above mentioned PDE polypeptide with an amino-terminal deletion and improved physicochemical characteristics can be any isoform
15 of PDE. For the most preferred embodiment the PDE polypeptide sequence is an isoform of PDE4D selected from the group consisting of D3, D4, D5, D6, D7, and D8. However, the present invention also extends to further isoforms of PDE that have not yet been identified.

Also of use to the present invention are mutants of the PDE polypeptide herein
20 before described. Of particular interest is a PDE polypeptide sequence comprising one or more mutations of Serine residues. Preferably, said Serines are mutated to either Alanine or Aspartic acid. In a most preferred embodiment, said Serine residues are selected from the group consisting of Ser54 and Ser579. Such Serines represent targets for
25 phosphorylation of PDE polypeptides. Phosphorylation of some of these sites has been shown to activate or inhibit the phosphodiesterase activity of PDEs.

Methods of generating amino acid substitutions in polypeptides are well known to those skilled in the art of Molecular Biology, e.g. methods such as site directed mutagenesis
30 as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (1989), Cold Spring Harbor Laboratory Press, New York, USA.

In another preferred embodiment of the present invention, the PDE polypeptide hereinbefore described exhibits decreased tubulin association, thus allowing to obtain purer PDE polypeptide preparations. To determine the decrease in tubulin association, the ratio of PDE molecules per tubulin molecules in the preparation of PDE polypeptide is calculated by quantitation of SDS-PAGE. Preferably, the ratio of PDE molecules per tubulin molecules is at least 5, preferably at least 10, more preferably at least 15, even more preferably at least 20, most preferably at least 25 molecules of PDE per molecules of tubulin. The tubulin content of PDE preparations is determined as follows: Equal volume aliquots of fractions from SEC are analyzed by SDS-PAGE (one gel per run and isoform, Figure 3). Optical densities of Coomassie stained PDE and tubulin bands are integrated as described hereinbefore for PDE aggregation quantitation. Integrated densities from PDE bands of fractions 2-11 are added up, giving a relative amount of total PDE, PDE_{total} . Likewise, integrated densities from tubulin bands of fractions 2-11 are added up, giving tubulin₂₋₁₁, and thereby the relative amount of total tubulin, tubulin_{total}. The ratio of PDE and tubulin (Figure 4C) is identical with the ratio of the relative amount of total PDE4D, PDE_{tot} , and relative amount of total tubulin, tubulin_{total}, on a mass per mass basis.

The polypeptide of this invention includes polypeptides that are substantially homologous or identical to the polypeptides shown in SEQ ID No. 1-7 and have, in their native form, the phosphodiesterase activity and specificity of PDE. In particular, the polypeptide of this invention also includes the previously published polypeptide sequences of PDE4D3 to D8 as defined in U50159 (D3), L20969 (D4), S:1059276 (D5) and the sequences for D6, D7 and D8 as disclosed in WO02/074992. As used herein, two polypeptides (or a region of the polypeptides) are substantially homologous or identical when the amino acid sequences are at least 45-55%, typically at least 70-75%, more typically at least 80-85%, and most typically greater than 90% or more homologous or identical. To determine the percent homology or identity of two amino acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of one polypeptide for optimal alignment with the other polypeptide or nucleic acid molecule). The amino acid residues or nucleotides at corresponding amino acid positions are then compared. When a position in one sequence is occupied by the same amino acid residue as the corresponding position in the other sequence, then the molecules are homologous at that position. As used herein, amino acid "homology" is equivalent to amino acid "identity". The percent homology between the two sequences is a

function of the number of identical positions shared by the sequences (*i.e.*, percent homology equals the number of identical positions/total number of positions times 100). The PDE polypeptide sequences of the present invention are preferably mammalian, most preferably human. The present invention also includes variants of the polypeptides
5 hereinbefore described with deletions, insertions, inversions, repeats, and substitutions selected according to general rules known in the art so as to have little effect on activity. For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, Science 247 (1990), 1306-1310, wherein the authors indicate that there are two main strategies for studying the tolerance of an amino acid
10 sequence to change.

The present invention provides polynucleotides that comprise polynucleotide sequences that encode the PDE polypeptide sequences hereinbefore described. As an example for a sequence comprised in such a polynucleotide, the polynucleotide sequence
15 encoding the "core construct" of PDE4D is shown in SEQ ID No. 8. However, the present invention relates to all polynucleotides comprising polynucleotide sequences encoding any of the PDE polypeptide sequences hereinbefore described.

The present invention provides an expression vector or virus comprising a
20 polynucleotide sequence that encodes the PDE polypeptide sequences hereinbefore described and which is capable of directing expression of the polynucleotide sequence in a compatible prokaryotic or eukaryotic host cell. The invention also provides a prokaryotic or eukaryotic host cell transformed or infected with such an expression vector or virus. The invention further provides a process for the production of such a polypeptide
25 comprising culturing the host cell expressing the PDE polypeptide sequences hereinbefore described in a suitable medium so that said polypeptide is expressed, and purifying said polypeptide from the cells. In a preferred embodiment, the virus used for the expression of a modified PDE polypeptide is a recombinant baculovirus. Methods of generating recombinant baculoviruses for expression of recombinant proteins are well known to the
30 man skilled in the art. Preferably, the recombinant baculovirus is based on pFASTBac as the transfer vector. The host cell of a preferred embodiment of the invention is an insect cell. Insect cells that can be infected with recombinant baculoviruses for the purpose of expressing recombinant proteins are well known in the art. The preferred process for the production of a modified PDE polypeptide is a process comprising culturing the insect cell

expressing the PDE polypeptide sequences hereinbefore described in a suitable medium so that said polypeptide is expressed, and purifying said polypeptide from the insect cell. To facilitate purification, the PDE polypeptide may also comprise a tag. Different types of such tags and the methods to join them with polynucleotide sequences encoding a polypeptide are well known to the person skilled in the art, e.g. myc-tag, FLAG-tag, six
5 Histidine tags (hereinafter referred to as 6xHis tag). A review on tagging of proteins can be found e.g. in Fritze and Anderson, *Methods in Enzymology* (2000) 327, 3-16. It is well known that such tags may be joined to either side of the polypeptide. Some tags are known which may also be removed enzymatically from the purified polypeptide, e.g. FLAG-tag
10 fused to the amino-terminus of a protein may be removed by digestion with bovine enterokinase (Hopp, T.P.(1988) *Biotechnology*, 6, 1204-1210). In a preferred embodiment the PDE polypeptides hereinbefore described are linked at their C-terminus to a 6xHis tag. The use of such 6xHis tags for ease of purification of recombinant polypeptides, and methods of purification of 6xHis tagged polypeptides are well known (e.g. in Sisk et al.
15 (1994) *J. Virol.* 68, 766, and Petty, K.J. (1996) in: Ausubel, F.M. et al., eds. *Current Protocols in Molecular Biology*, Vol. 2, New York: John Wiley and Sons).

In a preferred embodiment, the polypeptide of the present invention is purified. As used herein, a polypeptide is said to be "purified" when it is substantially free of cellular
20 material when it is isolated from recombinant and non-recombinant cells. A polypeptide, however, can be joined to another polypeptide with which it is not normally associated in a cell (e.g. in a "fusion protein") and still be "purified". Preferably, the polypeptides of the present invention are purified. The polypeptides of the invention can be purified to homogeneity. It is understood, however, that preparations in which the polypeptide is not
25 purified to homogeneity are useful. The critical feature is that the preparation allows for the desired function of the polypeptide, even in the presence of considerable amounts of other components. Thus, the invention encompasses various degrees of purity. In one embodiment, the language "substantially free of cellular material" includes preparations of the polypeptide having less than about 30% (by dry weight) other proteins (i.e.,
30 contaminating protein), less than about 20% other proteins, less than about 10% other proteins, or less than about 5% other proteins. When a polypeptide is recombinantly produced, it can also be substantially free of culture medium, i.e., culture medium represents less than about 20%, less than about 10%, or less than about 5% of the volume of the polypeptide preparation.

The invention further provides an antibody against any polypeptide hereinbefore described. Preferably, the antibody binds to an epitope of the N-terminal domain of the PDE polypeptides. In another preferred embodiment the antibody binds to the N-terminus of a PDE4D polypeptide selected from the group consisting of D3, D4, D5, D6, D7, and D8. Such antibodies may be useful for different purposes. They may be used for detection of the polypeptides of this invention. They may also be used for affinity purification of the polypeptides of the present invention. Furthermore, they may be used in a screening assay to identify agonists or antagonists of PDE4D phosphodiesterase activity.

10

The polypeptides of the present invention can be used for a process of identifying and obtaining a drug candidate for therapy of a vascular disorder, said process comprising measuring the activation or inhibition of the phosphodiesterase activity of any of the above mentioned polypeptides. Different methods for measuring phosphodiesterase activity are known, eg. from Owens et al., Biochem. J. (1997) 326, 53-60. Such a drug candidate may be a compound that is either an agonist or an antagonist of the phosphodiesterase activity of PDE.

The present invention provides a pharmaceutical composition comprising an activator or inhibitor of the phosphodiesterase activity of the PDE polypeptide of the present invention, and a pharmaceutically acceptable carrier. Any conventional carrier material can be utilized. The carrier material can be an organic or inorganic one suitable for eteral, percutaneous or parenteral administration. Suitable carriers include water, gelatin, gum arabic, lactose, starch, magnesium stearate, talc, vegetable oils, polyalkylene-glycols, petroleum jelly and the like. Furthermore, the pharmaceutical preparations may contain other pharmaceutically active agents. Additional additives such as flavoring agents, stabilizers, emulsifying agents, buffers and the like may be added in accordance with accepted practices of pharmaceutical compounding.

30 An agonist or antagonist of the phosphodiesterase activity of the PDE polypeptide of the present invention or a pharmaceutically acceptable salt thereof may be used for the preparation of a medicament for the treatment of diseases caused by elevated or reduced phosphodiesterase activities. Such diseases include asthma, atopic dermatitis, depression,

septic shock, toxic shock, autoimmune diabetes, AIDS, Crohn's disease, multiple sclerosis, psoriasis, allograft rejection, ulcerative colitis, cachexia, cerebral malaria, allergic rhinoconjunctivitis, osteoarthritis, rheumatoid arthritis, autoimmune encephalomyelitis, and vascular diseases such as reperfusion injury, cerebral ischemia, restenosis, stroke or
5 peripheral arterial occlusive disease. The invention is, however, not limited to the diseases listed hereinbefore, but extends to all diseases that depend on elevated or reduced phosphodiesterase activity. Of particular interest to this invention is the use of such an agonist or antagonist of phosphodiesterase activity of PDE or a pharmaceutically acceptable salt thereof for the preparation of a medicament for the treatment of a vascular
10 disease. The most preferred embodiment is the use of such agonist or antagonist of the phosphodiesterase activity of PDE or a pharmaceutically acceptable salt thereof for the preparation of a medicament for the treatment of stroke.

The present invention also provides a screening assay for the identification of
15 agonists or antagonists of phosphodiesterase activity comprising at least one of the above described polypeptides. In addition, the polypeptides hereinbefore described can be used for crystallization. Crystals of the polypeptides are useful for assaying the interaction of compounds with phosphodiesterases. Thus, the present invention also provides a method of assaying a candidate agonist or antagonist for its ability to interact with a
20 phosphodiesterase comprising crystallizing the polypeptide of claims 1 to 13 in a condition suitable for X-ray crystallography and conducting said X-ray crystallography on said polypeptide.

Furthermore, the present invention provides a kit for identifying agonists or
25 antagonists of phosphodiesterase activity comprising at least one of the polypeptides hereinbefore described.

In the present invention, the products, methods and uses substantially as herein before described, especially with reference to the foregoing examples, are also claimed.

30

The invention will be further described by the following non-limiting examples.

Examples

In the following the term "isoform" relates to the natural PDE4D splice variants D3 and D5-D8 and the invented core variant, DC.

5

Cloning of the PDE4D isoforms 3-8 and the core construct

Core construct:

A cDNA encoding the core fragment that is common for all the PDE4D isoforms 3-8 starting with the amino acid sequence FDV carboxyterminal to the LF1 splice site was generated by PCR using a 5' oligonucleotide with a HindIII cloning site (gatgaattcaagcttttgatgtggacaatggcaca) introducing two additional amino acid (K and L) in front of the FDV sequence. At the 3' end a set of primers was used that either generated the native sequence (gtgatatctcattatcacgtgtcaggagaacgatcatctatgaca) or added a sequence encoding 6xHis residues (gtgatatctcattatcaa tgggatggtgatggtgcgtgtcaggagaacgatcatctatgac) to enable rapid purification of the recombinant proteins. The cDNA encoding the core construct was cloned as a EcoRI-EcoRV fragment into the expression vector pENTRTM1a (GIBCO/BRL)

15

Isoforms (except core construct):

The DNA fragments encoding the isoform specific N-termini were generated by using synthetic oligonucleotides with terminal restriction enzyme sites for EcoRI and HindIII incorporated for directional cloning. These isoform specific DNA fragments were fused to the core construct sequence via the HindIII site introducing two additional amino acid residues (K and L). The integrity of the clones was confirmed by DNA sequencing prior to expression.

25

Expression of the PDE4D isoforms and the core construct in insect cells

The cDNAs were cloned into the pFASTBAC1 vector (Life Technologies. Inc) for expression in insect cells and the products were confirmed by sequencing. After recombination into the baculovirus genome the purified viral DNAs were transformed into the insect cells. Sf9 cells were cultured at 27°C in TC100 medium (BioWhittaker) with

30

5% (v/v) fetal calf serum. Virus stocks were generated with a titer of 1.5×10^9 pfu/ml. For large scale production of the isoforms 1-24 L fermentations of Sf9 cells were infected with a MOI of 1.

5 In one example, the 6xHis tagged PDE4D polypeptides DC, D3, D5, D6, D7 and D8 were produced in Sf9 cells in 1L spinner flasks using SF1 medium in the absence of serum. Infected cells were harvested 3 days after infection with the recombinant baculoviruses.

10 In another example, the PDE4D core construct DC was produced in a 24 L Airlifter Fermenter with 15 L medium (SF1 with 0% serum), 0.15 L lipids and 9 L Sf9 cells. During the entire fermentation procedure the cells were cultured at pH 6.2, $27.0 \pm 0.2^\circ\text{C}$ and a pO_2 of 30.0 ± 0.5 %. Cells were grown for 3 days. The cell number at infection was 2.3×10^6 cells/ml. Cells were infected with 450 ml recombinant baculovirus. Cells were harvested at 68h post infection and the cell pellet as well as the concentrated supernatant
15 stored at -80°C until further processing.

Purification of 6xHis tagged PDE4D isoforms

Sf9 cells from 1 liter culture broth, overexpressing the respective isoform, were resuspended on ice in 50 ml 50 mM HEPES pH 7.8, 300 mM NaCl 10 mM imidazole, 1
20 mM DTT, supplemented with protease inhibitors (one protease inhibitor cocktail tablet "complete, EDTA-free"; Roche). Opening of cells was performed by use of a 50 ml Dounce homogenizator and the homogeneous mixture was centrifuged for 1 hour at $70'000 \text{ g}$ and 4°C (Kontron TFT 45.94 rotor at $30'000 \text{ rpm}$). The supernate was filtrated through a filter with a pore size of $1.2 \mu\text{M}$ (Minisart; Sartorius, Germany) and then applied to a 6 ml Ni-
25 NTA agarose column at 2 ml/min. After equilibration with 50 mM HEPES pH 7.8, 300 mM NaCl, 10 mM imidazole, protein was eluted with a linear 30 ml gradient from 10 to 230 mM imidazole in the same buffer. Fractions containing the PDE4D isoform as analyzed by Coomassie stained SDS-PAGE were pooled and stored frozen at -80°C . Fresh Ni-NTA agarose material was used for every different PDE4D isoform preparation in
30 order to prevent cross contamination of isoforms.

Specific activities of 6xHis tagged PDE4D isoforms

Relative concentrations of Ni-NTA agarose purified isoform preparations were estimated by SDS-PAGE. Equal volume amounts of isoform preparations were applied to a gradient gel (4-12% NuPage; Invitrogen). After electrophoresis the Coomassie stained polyacrylamide gel was imaged by a video imaging system (Figure 1). Optical densities of PDE4D bands were integrated using a Macintosh computer and the public domain software "NIH Image", version 1.61 (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). The integrated arbitrary units per PDE4D band as returned by the software reflect the relative PDE4D concentrations within the original pools (Figure 1, red bars). Identities of PDE4D and tubulin bands had been verified by independent SDS-PAGE, excision of corresponding bands, trypsin cleavage and identification of tryptic peptides by MALDI-MS.

Activities of equal volume amounts of 10^6 -fold diluted purified isoforms were determined by use of a commercial radioactive phosphodiesterase assay (cAMP-dependent phosphodiesterase [^3H] assay; Amersham Pharmacia Biotech), following the instructions of the manufacturer. The obtained arbitrary activity units reflect the relative PDE4D activities within the original pools (Figure 1, green bars).

Relative specific activities of PDE4D isoforms were calculated by dividing relative activity values by relative concentration values (Figure 1, blue bars).

20

Qualitative investigation of aggregation by size exclusion chromatography (SEC)

50 μl of Ni-NTA agarose purified isoform preparation was injected into a Superose 12 size exclusion column (type PC3.2/30; Amersham Pharmacia Biotech), equilibrated with 50 mM TrisHCl pH 7.7, 100 mM NaCl, 0.5 mM MgCl_2 at a flow rate of 0.1 ml/min at 4°C. Chromatograms were recorded at 278 nm. Starting from the elution volume, the column eluate was collected as 50 μl fractions (Figure 2).

25

Quantification of aggregates and tubulin content

Equal volume aliquots of fractions from SEC were analyzed by SDS-PAGE (one gel per run and isoform, Figure 3). Optical densities of Coomassie stained PDE4D and tubulin bands were integrated as described above. Integrated densities from PDE4D bands of

30

fractions 2-4 were added up, giving a relative amount of aggregated PDE4D, PDE_{agg} (Figure 3). Similarly, integrated densities from PDE4D bands of fractions 5-11 were added up, giving a relative amount of non-aggregated PDE4D, $PDE_{non-agg}$. Relative amount of total PDE4D, PDE_{total} , is given by the sum of PDE_{agg} and $PDE_{non-agg}$. The percentage of non-aggregated PDE is given by the ratio of $PDE_{non-agg}$ and PDE_{total} , multiplied by 100 (Figure 4B).

Similarly, integrated densities from tubulin bands of fractions 2-11 were added up, giving tubulin₂₋₁₁, and thereby the relative amount of total tubulin, tubulin_{total}. The ratio of PDE and tubulin (Figure 4C) is identical with the ratio of relative amount of total PDE4D, PDE_{tot} , and relative amount of total tubulin, tubulin_{total}.

Activity assay and inhibition of phosphodiesterase activity

An IMAP FP-Assay was used for the determination of phosphodiesterase activity. The phosphodiesterase activity of the core construct and PDE4D3 was measured using the HEFP Phosphodiesterase Assay Kit (Molecular Devices). 2 µl of PDE4D3 or PDE4D core construct, 2 µl of cAMP (to a final concentration of 40 nM) and 1 µl of test substance or carrier were incubated for 45 min on a shaker. 12 µl of Binding Solution provided by the kit (with beads diluted 1:320) were added, and the reaction mixture incubated on a shaker for 2 hours. Fluorescence polarisation of the samples was measured in a Packard BioScience Fusion a-FP HT using as an emission filter a Polarizer 535, and as an excitation filter, Fluorescein 485/20. Inhibition of the phosphodiesterase activity of the PDE4D core construct was determined using Rolipram as inhibitor, and using PDE4D core construct at 30 ng/ml.

Claims

1. A polypeptide comprising a PDE polypeptide sequence with an amino-terminal deletion, said polypeptide exhibiting decreased aggregate formation.
- 5 2. The polypeptide of claim 1, wherein said PDE polypeptide sequence is a long form PDE polypeptide sequence.
3. The polypeptide of claims 1 or 2, wherein said PDE polypeptide sequence is a PDE4 polypeptide sequence.
- 10 4. The polypeptide of any of claims 1 to 3 wherein said PDE polypeptide sequence is a PDE4D polypeptide sequence.
- 15 5. The polypeptide of any of claims 1 to 4 wherein the proportion of non-aggregated polypeptide in the total polypeptide preparation is from 55% to 100% of total polypeptide.
- 20 6. The polypeptide of any of claims 1 to 6, wherein the polypeptide sequence starts at any amino acid located between the LF1 splice site and the first amino acid of the UCR1 start of the native PDE polypeptide.
7. The polypeptide of any of claims 1 to 7, wherein the polypeptide sequence starts at the LF1 splice site of the native PDE polypeptide.
- 25 8. The polypeptide of any of claims 1 to 8, wherein the PDE polypeptide sequence start is located 13 amino acids upstream of the UCR1 start of the native PDE polypeptide.

9. The polypeptide of any of claims 4 to 7 wherein the PDE4D polypeptide sequence is a sequence of an isoform selected from the group consisting of D3, D4, D5, D6, D7, and D8.

5

10. The polypeptide of claims 1 to 9 wherein said polypeptide comprises one or more mutations of Serine residues.

- 10 11. The polypeptide of claim 10 wherein said Serine residues are mutated to either Alanine or Aspartic acid.

12. The polypeptide of claims 10 or 11 wherein said Serine residues are selected from the group consisting of Ser54 and Ser579.

- 15 13. The polypeptide of any one of claims 1 to 9 wherein said polypeptide exhibits decreased tubulin association.

14. A polynucleotide sequence encoding a polypeptide as claimed in claims 1 to 13.

- 20 15. An expression vector or virus comprising a polynucleotide sequence as claimed in claim 14 and being capable of directing expression of the polynucleotide sequence in a compatible prokaryotic or eukaryotic host cell.

16. A virus as claimed in claim 15 wherein the virus is a recombinant baculovirus.

25

17. A prokaryotic or eukaryotic host cell transformed or infected with the expression vector or virus of claims 15 or 16.

18. A host cell as claimed in claim 17, wherein the host cell is an insect cell.

5

19. A process for the production of a polypeptide as claimed in claims 1 to 13, comprising culturing the host cell of claims 15 or 16 in a suitable medium so that said polypeptide is expressed, and purifying said polypeptide from the cells.

10 20. A process for the production of a polypeptide as claimed in claim 19 wherein the host cell is an insect cell.

21. A polypeptide according to claims 1 to 13 produced by the process of claims 19 or 20.

15 22. An antibody against a polypeptide as claimed in claims 1 to 13.

23. An antibody as claimed in claim 22, wherein the antibody binds to an epitope of the N-terminal domain of the polypeptide of claims 1 to 13.

20 24. A process for identifying and obtaining a drug candidate for therapy of an inflammatory disease, said process comprising measuring the activation or inhibition of the phosphodiesterase activity of any of the polypeptides claimed in claims 1 to 13.

25. A compound identified according to the process of claim 24.

25

26. A pharmaceutical composition comprising an activator or inhibitor of the phosphodiesterase activity of the polypeptide claimed in any of the claims 1 to 13, and a pharmaceutically acceptable carrier.
- 5 27. Use of an activator or inhibitor of the phosphodiesterase activity of the polypeptide claimed in any of the claims 1 to 13 or a pharmaceutically acceptable salt thereof for the preparation of a medicament for the treatment of an inflammatory disease.
- 10 28. A screening assay for the identification of agonists or antagonists of phosphodiesterase activity comprising at least one of the polypeptides of claims 1 to 13.
29. Use of a polypeptide of claims 1 to 13 for crystallization.
- 15 30. A method of assaying a candidate agonist or antagonist for its ability to interact with a phosphodiesterase comprising crystallizing a polypeptide of claims 1 to 13 in a condition suitable for X-ray crystallography and conducting said X-ray crystallography on said polypeptide.
- 20 31. A kit for identifying agonists or antagonists of phosphodiesterase activity comprising at least one of the polypeptides of claims 1 to 13.
32. The products, methods and uses substantially as herein before described, especially with reference to the foregoing examples.

Abstract

Modified phosphodiesterase (PDE) polypeptides with altered physicochemical properties are disclosed, together with the polynucleotides encoding such modified PDE
5 polypeptides, antibodies against such polypeptides and methods of preparation. Such polypeptides are useful for screening assays for identification of agonists or antagonists of phosphodiesterase activity.

This Page Blank (uspto)

Figure 1

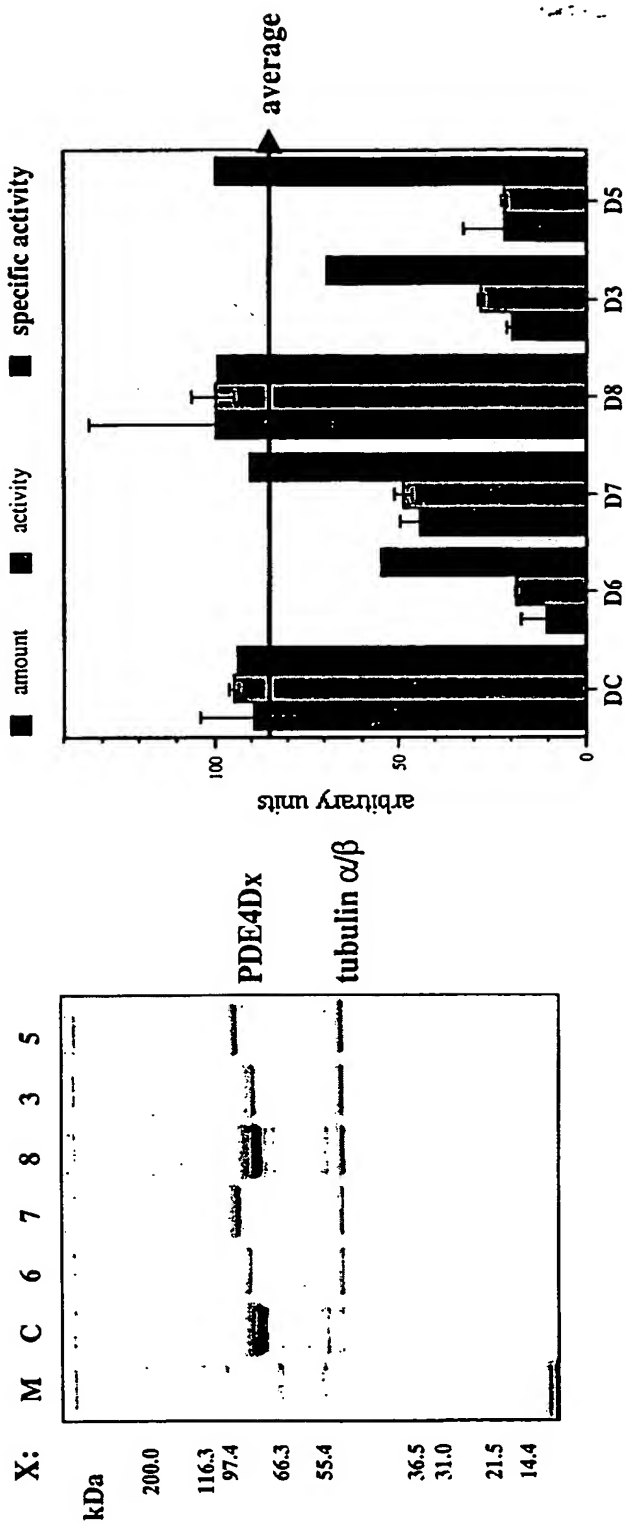


Figure 2

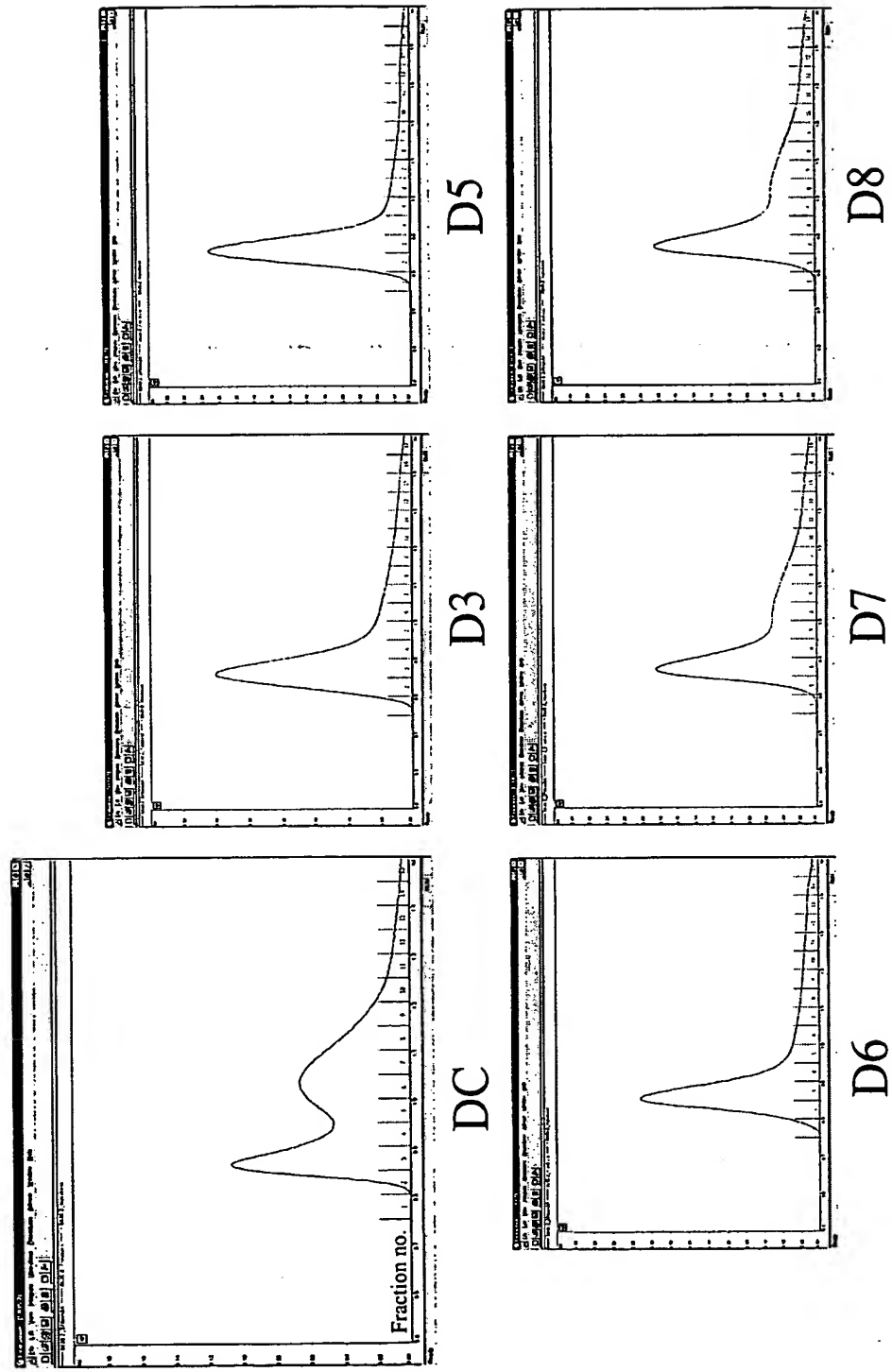


Figure 3

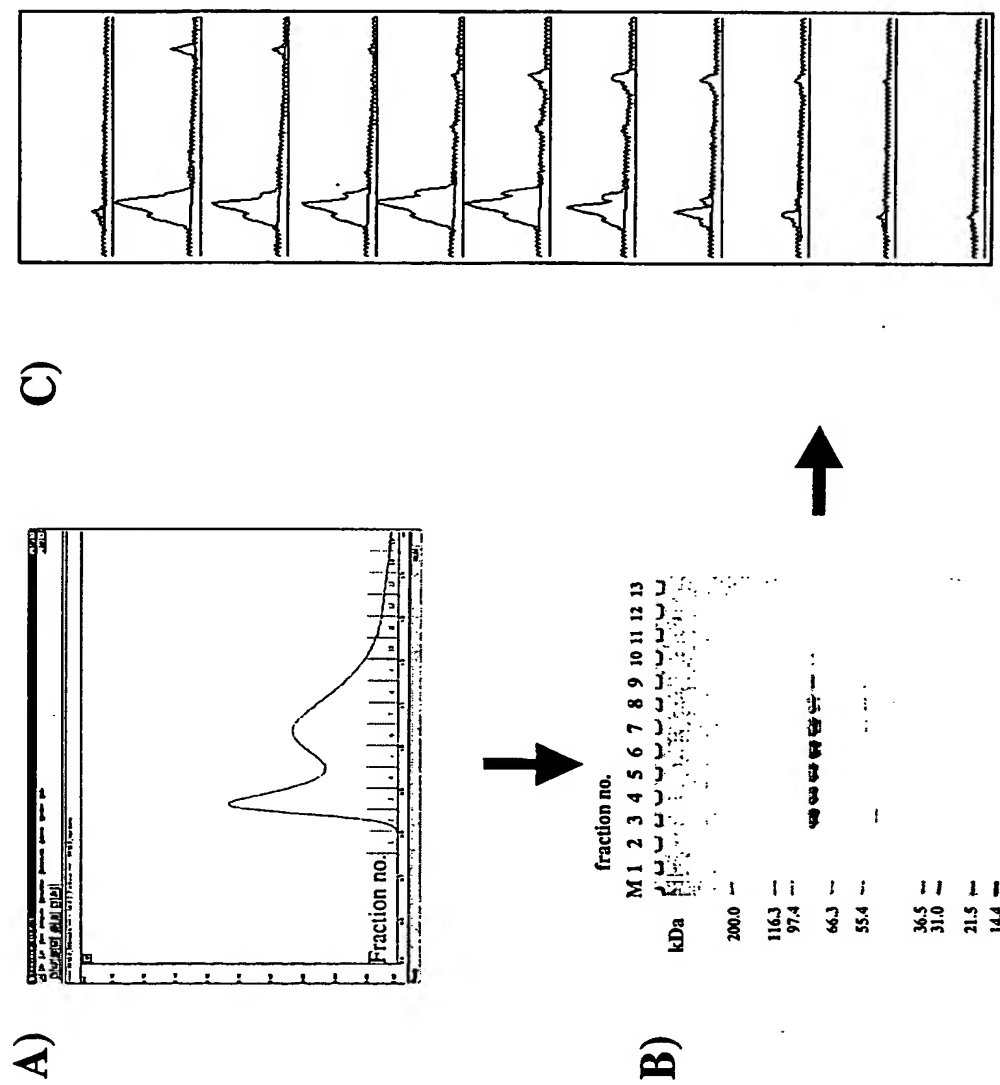
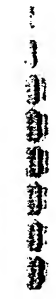


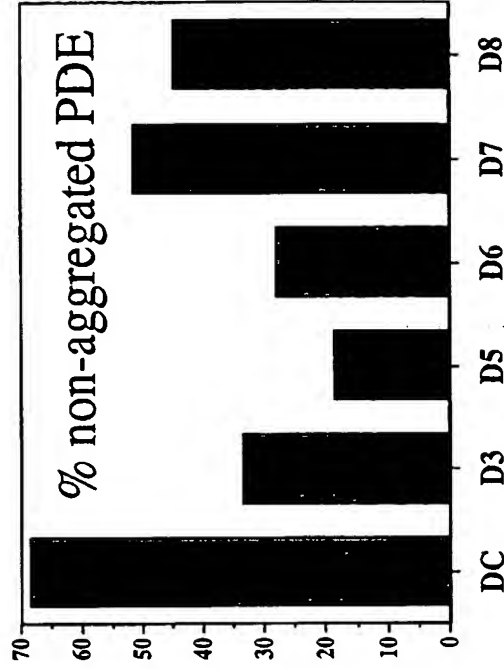
Figure 4

Fraction No. 2 3 4 5 6 7 8 9 10 11

A)



B)



C)

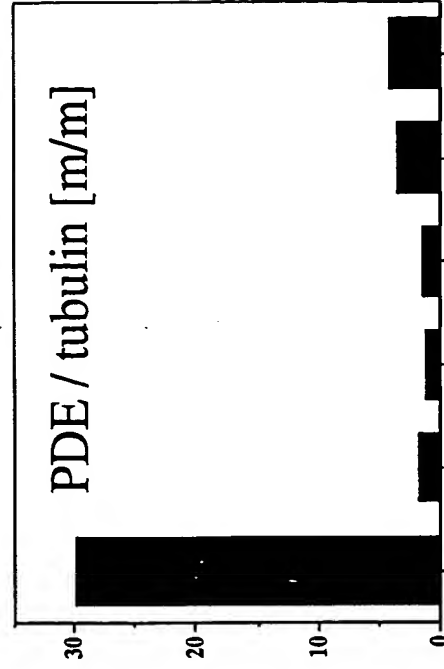


Figure 5

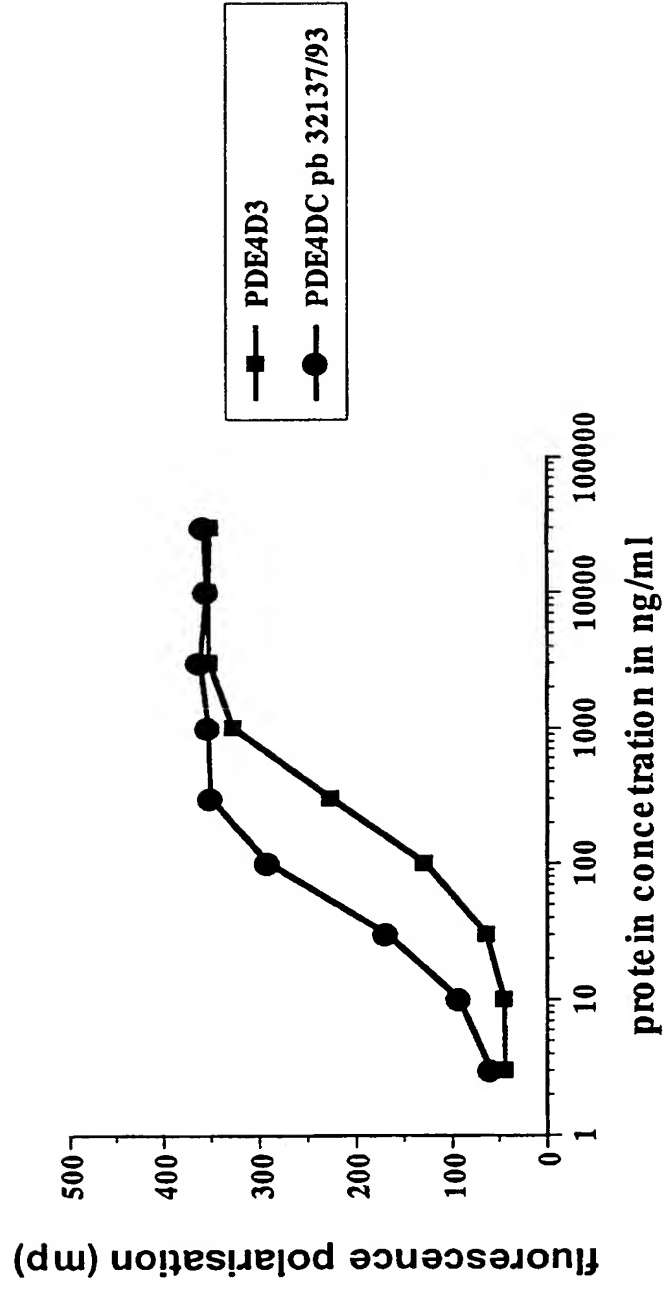
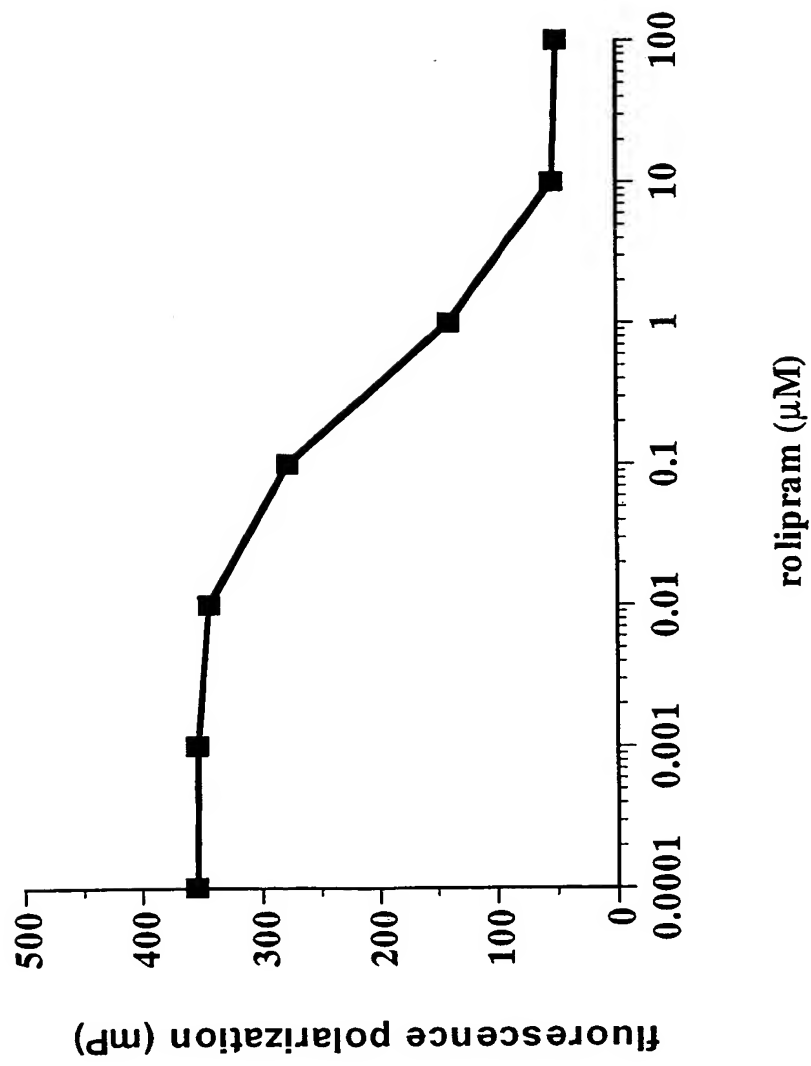


Figure 6

**fluorescence polarization assay
30ng/ml PDE4D core construct IC₅₀ Rolipram @40nMcAMP**



17. Dez. 2002

SEQUENCE LISTING

<110> F. Hoffmann-La Roche AG

5 <120> Modified PDE4D polypeptides

<130> case 21507

<160> 6

<170> PatentIn version 3.1

<210> 1

10 <211> 673

<212> PRT

<213> Homo sapiens

<220>

<221> PDE4D3

15 <222> (1)..(673)

<223>

<220>

<221> LF1_splice_site

<222> (17)..(17)

20 <223>

<220>

<221> Ser579

<222> (579)..(579)

<223>

25 <220>

<221> Ser54

<222> (54) .. (54)

<223>

<220>

<221> UCR1_site_-13

5 <222> (37) .. (37)

<223>

<220>

<221> UCR1_start

<222> (50) .. (50)

10 <223>

<400> 1

Met Met His Val Asn Asn Phe Pro Phe Arg Arg His Ser Trp Ile Cys

1 5 10 15

Phe Asp Val Asp Asn Gly Thr Ser Ala Gly Arg Ser Pro Leu Asp Pro

15 20 25 30

Met Thr Ser Pro Gly Ser Gly Leu Ile Leu Gln Ala Asn Phe Val His

35 40 45

Ser Gln Arg Arg Glu Ser Phe Leu Tyr Arg Ser Asp Ser Asp Tyr Asp

50 55 60

20 Leu Ser Pro Lys Ser Met Ser Arg Asn Ser Ser Ile Ala Ser Asp Ile

65 70 75 80

His Gly Asp Asp Leu Ile Val Thr Pro Phe Ala Gln Val Leu Ala Ser

85 90 95

Leu Arg Thr Val Arg Asn Asn Phe Ala Ala Leu Thr Asn Leu Gln Asp

25 100 105 110

Arg Ala Pro Ser Lys Arg Ser Pro Met Cys Asn Gln Pro Ser Ile Asn
115 120 125

Lys Ala Thr Ile Thr Glu Glu Ala Tyr Gln Lys Leu Ala Ser Glu Thr
130 135 140

5 Leu Glu Glu Leu Asp Trp Cys Leu Asp Gln Leu Glu Thr Leu Gln Thr
145 150 155 160

Arg His Ser Val Ser Glu Met Ala Ser Asn Lys Phe Lys Arg Met Leu
165 170 175

Asn Arg Glu Leu Thr His Leu Ser Glu Met Ser Arg Ser Gly Asn Gln
10 180 185 190

Val Ser Glu Phe Ile Ser Asn Thr Phe Leu Asp Lys Gln His Glu Val
195 200 205

Glu Ile Pro Ser Pro Thr Gln Lys Glu Lys Glu Lys Lys Lys Arg Pro
210 215 220

15 Met Ser Gln Ile Ser Gly Val Lys Lys Leu Met His Ser Ser Ser Leu
225 230 235 240

Thr Asn Ser Ser Ile Pro Arg Phe Gly Val Lys Thr Glu Gln Glu Asp
245 250 255

Val Leu Ala Lys Glu Leu Glu Asp Val Asn Lys Trp Gly Leu His Val
20 260 265 270

Phe Arg Ile Ala Glu Leu Ser Gly Asn Arg Pro Leu Thr Val Ile Met
275 280 285

His Thr Ile Phe Gln Glu Arg Asp Leu Leu Lys Thr Phe Lys Ile Pro
290 295 300

25 Val Asp Thr Leu Ile Thr Tyr Leu Met Thr Leu Glu Asp His Tyr His

Glu Arg Glu Arg Gly Met Glu Ile Ser Pro Met Cys Asp Lys His Asn
515 520 525
Ala Ser Val Glu Lys Ser Gln Val Gly Phe Ile Asp Tyr Ile Val His
530 535 540
5 Pro Leu Trp Glu Thr Trp Ala Asp Leu Val His Pro Asp Ala Gln Asp
545 550 555 560
Ile Leu Asp Thr Leu Glu Asp Asn Arg Glu Trp Tyr Gln Ser Thr Ile
565 570 575
Pro Gln Ser Pro Ser Pro Ala Pro Asp Asp Pro Glu Glu Gly Arg Gln
10 580 585 590
Gly Gln Thr Glu Lys Phe Gln Phe Glu Leu Thr Leu Glu Glu Asp Gly
595 600 605
Glu Ser Asp Thr Glu Lys Asp Ser Gly Ser Gln Val Glu Glu Asp Thr
610 615 620
15 Ser Cys Ser Asp Ser Lys Thr Leu Cys Thr Gln Asp Ser Glu Ser Thr
625 630 635 640
Glu Ile Pro Leu Asp Glu Gln Val Glu Glu Glu Ala Val Gly Glu Glu
645 650 655
Glu Glu Ser Gln Pro Glu Ala Cys Val Ile Asp Asp Arg Ser Pro Asp
20 660 665 670
Thr

<210> 2

25 <211> 664

<212> PRT

<213> Homo sapiens

<220>

<221> PDE4D_core

5 <222> (1) .. (664)

<223>

<220>

<221> LF1_splice_site

<222> (2) .. (2)

10 <223>

<220>

<221> UCR1_site

<222> (35) .. (35)

<223>

15 <220>

<221> UCR1_site_-13

<222> (22) .. (22)

<223>

<220>

20 <221> SER54

<222> (39) .. (39)

<223>

<220>

<221> SER579

25 <222> (564) .. (564)

<223>

<400> 2

Met Phe Asp Val Asp Asn Gly Thr Ser Ala Gly Arg Ser Pro Leu Asp
1 5 10 15
5 Pro Met Thr Ser Pro Gly Ser Gly Leu Ile Leu Gln Ala Asn Phe Val
20 25 30
His Ser Gln Arg Arg Glu Ser Phe Leu Tyr Arg Ser Asp Ser Asp Tyr
35 40 45
Asp Leu Ser Pro Lys Ser Met Ser Arg Asn Ser Ser Ile Ala Ser Asp
10 50 55 60
Ile His Gly Asp Asp Leu Ile Val Thr Pro Phe Ala Gln Val Leu Ala
65 70 75 80
Ser Leu Arg Thr Val Arg Asn Asn Phe Ala Ala Leu Thr Asn Leu Gln
85 90 95
15 Asp Arg Ala Pro Ser Lys Arg Ser Pro Met Cys Asn Gln Pro Ser Ile
100 105 110
Asn Lys Ala Thr Ile Thr Glu Glu Ala Tyr Gln Lys Leu Ala Ser Glu
115 120 125
Thr Leu Glu Glu Leu Asp Trp Cys Leu Asp Gln Leu Glu Thr Leu Gln
20 130 135 140
Thr Arg His Ser Val Ser Glu Met Ala Ser Asn Lys Phe Lys Arg Met
145 150 155 160
Leu Asn Arg Glu Leu Thr His Leu Ser Glu Met Ser Arg Ser Gly Asn
165 170 175
25 Gln Val Ser Glu Phe Ile Ser Asn Thr Phe Leu Asp Lys Gln His Glu

	180	185	190
	Val Glu Ile Pro Ser Pro Thr Gln Lys Glu Lys Glu Lys Lys Lys Arg		
	195	200	205
	Pro Met Ser Gln Ile Ser Gly Val Lys Lys Leu Met His Ser Ser Ser		
5	210	215	220
	Leu Thr Asn Ser Ser Ile Pro Arg Phe Gly Val Lys Thr Glu Gln Glu		
	225	230	235
	Asp Val Leu Ala Lys Glu Leu Glu Asp Val Asn Lys Trp Gly Leu His		
	245	250	255
10	Val Phe Arg Ile Ala Glu Leu Ser Gly Asn Arg Pro Leu Thr Val Ile		
	260	265	270
	Met His Thr Ile Phe Gln Glu Arg Asp Leu Leu Lys Thr Phe Lys Ile		
	275	280	285
	Pro Val Asp Thr Leu Ile Thr Tyr Leu Met Thr Leu Glu Asp His Tyr		
15	290	295	300
	His Ala Asp Val Ala Tyr His Asn Asn Ile His Ala Ala Asp Val Val		
	305	310	315
	Gln Ser Thr His Val Leu Leu Ser Thr Pro Ala Leu Glu Ala Val Phe		
	325	330	335
20	Thr Asp Leu Glu Ile Leu Ala Ala Ile Phe Ala Ser Ala Ile His Asp		
	340	345	350
	Val Asp His Pro Gly Val Ser Asn Gln Phe Leu Ile Asn Thr Asn Ser		
	355	360	365
	Glu Leu Ala Leu Met Tyr Asn Asp Ser Ser Val Leu Glu Asn His His		
25	370	375	380

Leu Ala Val Gly Phe Lys Leu Leu Gln Glu Glu Asn Cys Asp Ile Phe
385 390 395 400
Gln Asn Leu Thr Lys Lys Gln Arg Gln Ser Leu Arg Lys Met Val Ile
405 410 415
5 Asp Ile Val Leu Ala Thr Asp Met Ser Lys His Met Asn Leu Leu Ala
420 425 430
Asp Leu Lys Thr Met Val Glu Thr Lys Lys Val Thr Ser Ser Gly Val
435 440 445
Leu Leu Leu Asp Asn Tyr Ser Asp Arg Ile Gln Val Leu Gln Asn Met
10 450 455 460
Val His Cys Ala Asp Leu Ser Asn Pro Thr Lys Pro Leu Gln Leu Tyr
465 470 475 480
Arg Gln Trp Thr Asp Arg Ile Met Glu Glu Phe Phe Arg Gln Gly Asp
485 490 495
15 Arg Glu Arg Glu Arg Gly Met Glu Ile Ser Pro Met Cys Asp Lys His
500 505 510
Asn Ala Ser Val Glu Lys Ser Gln Val Gly Phe Ile Asp Tyr Ile Val
515 520 525
His Pro Leu Trp Glu Thr Trp Ala Asp Leu Val His Pro Asp Ala Gln
20 530 535 540
Asp Ile Leu Asp Thr Leu Glu Asp Asn Arg Glu Trp Tyr Gln Ser Thr
545 550 555 560
Ile Pro Gln Ser Pro Ser Pro Ala Pro Asp Asp Pro Glu Glu Gly Arg
565 570 575
25 Gln Gly Gln Thr Glu Lys Phe Gln Phe Glu Leu Thr Leu Glu Glu Asp

580 585 590
Gly Glu Ser Asp Thr Glu Lys Asp Ser Gly Ser Gln Val Glu Glu Asp
595 600 605
Thr Ser Cys Ser Asp Ser Lys Thr Leu Cys Thr Gln Asp Ser Glu Ser
5 610 615 620
Thr Glu Ile Pro Leu Asp Glu Gln Val Glu Glu Glu Ala Val Gly Glu
625 630 635 640
Glu Glu Glu Ser Gln Pro Glu Ala Cys Val Ile Asp Asp Arg Ser Pro
645 650 655
10 Asp Thr His His His His His His
660

<210> 3
<211> 747
15 <212> PRT
<213> Homo sapiens
<220>
<221> PDE4D5
<222> (1)..(747)
20 <223>
<220>
<221> isoform_specific_N-terminus
<222> (1)..(90)
<223>
25 <220>

<221> LF1_splice_site
<222> (91)..(91)
<223>
<220>

5 <221> UCR1_site
<222> (124)..(124)
<223>
<220>
<221> UCR1_site-13

10 <222> (111)..(111)
<223>
<220>
<221> Ser54
<222> (128)..(128)

15 <223>
<220>
<221> Ser579
<222> (653)..(653)
<223>

20 <400> 3

Met Ala Gln Gln Thr Ser Pro Asp Thr Leu Thr Val Pro Glu Val Asp
1 5 10 15
Asn Pro His Cys Pro Asn Pro Trp Leu Asn Glu Asp Leu Val Lys Ser
20 25 30
25 Leu Arg Glu Asn Leu Leu Gln His Glu Lys Ser Lys Thr Ala Arg Lys

	35	40	45
	Ser Val Ser Pro Lys Leu Ser Pro Val Ile Ser Pro Arg Asn Ser Pro		
	50	55	60
	Arg Leu Leu Arg Arg Met Leu Leu Ser Ser Asn Ile Pro Lys Gln Arg		
5	65	70	75 80
	Arg Phe Thr Val Ala His Thr Cys Lys Leu Phe Asp Val Asp Asn Gly		
	85	90	95
	Thr Ser Ala Gly Arg Ser Pro Leu Asp Pro Met Thr Ser Pro Gly Ser		
	100	105	110
10	Gly Leu Ile Leu Gln Ala Asn Phe Val His Ser Gln Arg Arg Glu Ser		
	115	120	125
	Phe Leu Tyr Arg Ser Asp Ser Asp Tyr Asp Leu Ser Pro Lys Ser Met		
	130	135	140
	Ser Arg Asn Ser Ser Ile Ala Ser Asp Ile His Gly Asp Asp Leu Ile		
15	145	150	155 160
	Val Thr Pro Phe Ala Gln Val Leu Ala Ser Leu Arg Thr Val Arg Asn		
	165	170	175
	Asn Phe Ala Ala Leu Thr Asn Leu Gln Asp Arg Ala Pro Ser Lys Arg		
	180	185	190
20	Ser Pro Met Cys Asn Gln Pro Ser Ile Asn Lys Ala Thr Ile Thr Glu		
	195	200	205
	Glu Ala Tyr Gln Lys Leu Ala Ser Glu Thr Leu Glu Glu Leu Asp Trp		
	210	215	220
	Cys Leu Asp Gln Leu Glu Thr Leu Gln Thr Arg His Ser Val Ser Glu		
25	225	230	235 240

Met Ala Ser Asn Lys Phe Lys Arg Met Leu Asn Arg Glu Leu Thr His
245 250 255

Leu Ser Glu Met Ser Arg Ser Gly Asn Gln Val Ser Glu Phe Ile Ser
260 265 270

5 Asn Thr Phe Leu Asp Lys Gln His Glu Val Glu Ile Pro Ser Pro Thr
275 280 285

Gln Lys Glu Lys Glu Lys Lys Lys Arg Pro Met Ser Gln Ile Ser Gly
290 295 300

Val Lys Lys Leu Met His Ser Ser Ser Leu Thr Asn Ser Ser Ile Pro
10 305 310 315 320

Arg Phe Gly Val Lys Thr Glu Gln Glu Asp Val Leu Ala Lys Glu Leu
325 330 335

Glu Asp Val Asn Lys Trp Gly Leu His Val Phe Arg Ile Ala Glu Leu
340 345 350

15 Ser Gly Asn Arg Pro Leu Thr Val Ile Met His Thr Ile Phe Gln Glu
355 360 365

Arg Asp Leu Leu Lys Thr Phe Lys Ile Pro Val Asp Thr Leu Ile Thr
370 375 380

Tyr Leu Met Thr Leu Glu Asp His Tyr His Ala Asp Val Ala Tyr His
20 385 390 395 400

Asn Asn Ile His Ala Ala Asp Val Val Gln Ser Thr His Val Leu Leu
405 410 415

Ser Thr Pro Ala Leu Glu Ala Val Phe Thr Asp Leu Glu Ile Leu Ala
420 425 430

25 Ala Ile Phe Ala Ser Ala Ile His Asp Val Asp His Pro Gly Val Ser

25 625 630 635 640

Asp Asn Arg Glu Trp Tyr Gln Ser Thr Ile Pro Gln Ser Pro Ser Pro

645

650

655

Ala Pro Asp Asp Pro Glu Glu Gly Arg Gln Gly Gln Thr Glu Lys Phe

660

665

670

5 Gln Phe Glu Leu Thr Leu Glu Glu Asp Gly Glu Ser Asp Thr Glu Lys

675

680

685

Asp Ser Gly Ser Gln Val Glu Glu Asp Thr Ser Cys Ser Asp Ser Lys

690

695

700

Thr Leu Cys Thr Gln Asp Ser Glu Ser Thr Glu Ile Pro Leu Asp Glu

10 705

710

715

720

Gln Val Glu Glu Glu Ala Val Gly Glu Glu Glu Glu Ser Gln Pro Glu

725

730

735

Ala Cys Val Ile Asp Asp Arg Ser Pro Asp Thr

740

745

15

<210> 4

<211> 689

<212> PRT

20 <213> Homo sapiens

<220>

<221> PDE4D6

<222> (1) .. (689)

<223>

25 <220>

<221> isoform_specific_N-terminus
<222> (1)..(32)
<223>
<220>

5 <221> LF1_splice_site
<222> (33)..(33)
<223>
<220>

10 <221> UCR1_start
<222> (66)..(66)
<223>
<220>

<221> UCR1_start-13
<222> (53)..(53)

15 <223>
<220>

<221> Ser54
<222> (70)..(70)
<223>

20 <220>

<221> Ser579
<222> (595)..(595)
<223>

<400> 4

25 Met Ala Phe Val Trp Asp Pro Leu Gly Ala Thr Val Pro Gly Pro Ser

1	5	10	15
Thr Arg Ala Lys Ser Arg Leu Arg Phe Ser Lys Ser Tyr Ser Lys Leu			
	20	25	30
Phe Asp Val Asp Asn Gly Thr Ser Ala Gly Arg Ser Pro Leu Asp Pro			
5	35	40	45
Met Thr Ser Pro Gly Ser Gly Leu Ile Leu Gln Ala Asn Phe Val His			
	50	55	60
Ser Gln Arg Arg Glu Ser Phe Leu Tyr Arg Ser Asp Ser Asp Tyr Asp			
65	70	75	80
10 Leu Ser Pro Lys Ser Met Ser Arg Asn Ser Ser Ile Ala Ser Asp Ile			
	85	90	95
His Gly Asp Asp Leu Ile Val Thr Pro Phe Ala Gln Val Leu Ala Ser			
	100	105	110
Leu Arg Thr Val Arg Asn Asn Phe Ala Ala Leu Thr Asn Leu Gln Asp			
15	115	120	125
Arg Ala Pro Ser Lys Arg Ser Pro Met Cys Asn Gln Pro Ser Ile Asn			
	130	135	140
Lys Ala Thr Ile Thr Glu Glu Ala Tyr Gln Lys Leu Ala Ser Glu Thr			
145	150	155	160
20 Leu Glu Glu Leu Asp Trp Cys Leu Asp Gln Leu Glu Thr Leu Gln Thr			
	165	170	175
Arg His Ser Val Ser Glu Met Ala Ser Asn Lys Phe Lys Arg Met Leu			
	180	185	190
Asn Arg Glu Leu Thr His Leu Ser Glu Met Ser Arg Ser Gly Asn Gln			
25	195	200	205

Val Ser Glu Phe Ile Ser Asn Thr Phe Leu Asp Lys Gln His Glu Val
210 215 220
Glu Ile Pro Ser Pro Thr Gln Lys Glu Lys Glu Lys Lys Lys Arg Pro
225 230 235 240
5 Met Ser Gln Ile Ser Gly Val Lys Lys Leu Met His Ser Ser Ser Leu
245 250 255
Thr Asn Ser Ser Ile Pro Arg Phe Gly Val Lys Thr Glu Gln Glu Asp
260 265 270
Val Leu Ala Lys Glu Leu Glu Asp Val Asn Lys Trp Gly Leu His Val
10 275 280 285
Phe Arg Ile Ala Glu Leu Ser Gly Asn Arg Pro Leu Thr Val Ile Met
290 295 300
His Thr Ile Phe Gln Glu Arg Asp Leu Leu Lys Thr Phe Lys Ile Pro
305 310 315 320
15 Val Asp Thr Leu Ile Thr Tyr Leu Met Thr Leu Glu Asp His Tyr His
325 330 335
Ala Asp Val Ala Tyr His Asn Asn Ile His Ala Ala Asp Val Val Gln
340 345 350
Ser Thr His Val Leu Leu Ser Thr Pro Ala Leu Glu Ala Val Phe Thr
20 355 360 365
Asp Leu Glu Ile Leu Ala Ala Ile Phe Ala Ser Ala Ile His Asp Val
370 375 380
Asp His Pro Gly Val Ser Asn Gln Phe Leu Ile Asn Thr Asn Ser Glu
385 390 395 400
25 Leu Ala Leu Met Tyr Asn Asp Ser Ser Val Leu Glu Asn His His Leu

	405	410	415
	Ala Val Gly Phe Lys Leu Leu Gln Glu Glu Asn Cys Asp Ile Phe Gln		
	420	425	430
	Asn Leu Thr Lys Lys Gln Arg Gln Ser Leu Arg Lys Met Val Ile Asp		
5	435	440	445
	Ile Val Leu Ala Thr Asp Met Ser Lys His Met Asn Leu Leu Ala Asp		
	450	455	460
	Leu Lys Thr Met Val Glu Thr Lys Lys Val Thr Ser Ser Gly Val Leu		
	465	470	475
10	Leu Leu Asp Asn Tyr Ser Asp Arg Ile Gln Val Leu Gln Asn Met Val		
	485	490	495
	His Cys Ala Asp Leu Ser Asn Pro Thr Lys Pro Leu Gln Leu Tyr Arg		
	500	505	510
	Gln Trp Thr Asp Arg Ile Met Glu Glu Phe Phe Arg Gln Gly Asp Arg		
15	515	520	525
	Glu Arg Glu Arg Gly Met Glu Ile Ser Pro Met Cys Asp Lys His Asn		
	530	535	540
	Ala Ser Val Glu Lys Ser Gln Val Gly Phe Ile Asp Tyr Ile Val His		
	545	550	555
20	Pro Leu Trp Glu Thr Trp Ala Asp Leu Val His Pro Asp Ala Gln Asp		
	565	570	575
	Ile Leu Asp Thr Leu Glu Asp Asn Arg Glu Trp Tyr Gln Ser Thr Ile		
	580	585	590
	Pro Gln Ser Pro Ser Pro Ala Pro Asp Asp Pro Glu Glu Gly Arg Gln		
25	595	600	605

Gly Gln Thr Glu Lys Phe Gln Phe Glu Leu Thr Leu Glu Glu Asp Gly
610 615 620
Glu Ser Asp Thr Glu Lys Asp Ser Gly Ser Gln Val Glu Glu Asp Thr
625 630 635 640
5 Ser Cys Ser Asp Ser Lys Thr Leu Cys Thr Gln Asp Ser Glu Ser Thr
645 650 655
Glu Ile Pro Leu Asp Glu Gln Val Glu Glu Glu Ala Val Gly Glu Glu
660 665 670
Glu Glu Ser Gln Pro Glu Ala Cys Val Ile Asp Asp Arg Ser Pro Asp
10 675 680 685
Thr

15 <210> 5
<211> 750
<212> PRT
<213> Homo sapiens
<220>
20 <221> PDE4D7
<222> (1)..(750)
<223>
<220>
<221> isoform_specific_N-terminus
25 <222> (1)..(93)

<223>

<220>

<221> LF1_splice_site

<222> (94)..(94)

5 <223>

<220>

<221> UCR1_start

<222> (127)..(127)

<223>

10 <220>

<221> UCR1_start-13

<222> (114)..(114)

<223>

<220>

15 <221> Ser54

<222> (131)..(131)

<223>

<220>

<221> Ser579

20 <222> (656)..(656)

<223>

<400> 5

Met Lys Arg Asn Thr Cys Asp Leu Leu Ser Arg Ser Lys Ser Ala Ser

1

5

10

15

25 Glu Glu Thr Leu His Ser Ser Asn Glu Glu Glu Asp Pro Phe Arg Gly

	-20	25	30
	Met Glu Pro Tyr Leu Val Arg Arg Leu Ser Cys Arg Asn Ile Gln Leu		
	35	40	45
	Pro Pro Leu Ala Phe Arg Gln Leu Glu Gln Ala Asp Leu Lys Ser Glu		
5	50	55	60
	Ser Glu Asn Ile Gln Arg Pro Thr Ser Leu Pro Leu Lys Ile Leu Pro		
	65	70	75 80
	Leu Ile Ala Ile Thr Ser Ala Glu Ser Ser Gly Lys Leu Phe Asp Val		
	85	90	95
10	Asp Asn Gly Thr Ser Ala Gly Arg Ser Pro Leu Asp Pro Met Thr Ser		
	100	105	110
	Pro Gly Ser Gly Leu Ile Leu Gln Ala Asn Phe Val His Ser Gln Arg		
	115	120	125
	Arg Glu Ser Phe Leu Tyr Arg Ser Asp Ser Asp Tyr Asp Leu Ser Pro		
15	130	135	140
	Lys Ser Met Ser Arg Asn Ser Ser Ile Ala Ser Asp Ile His Gly Asp		
	145	150	155 160
	Asp Leu Ile Val Thr Pro Phe Ala Gln Val Leu Ala Ser Leu Arg Thr		
	165	170	175
20	Val Arg Asn Asn Phe Ala Ala Leu Thr Asn Leu Gln Asp Arg Ala Pro		
	180	185	190
	Ser Lys Arg Ser Pro Met Cys Asn Gln Pro Ser Ile Asn Lys Ala Thr		
	195	200	205
	Ile Thr Glu Glu Ala Tyr Gln Lys Leu Ala Ser Glu Thr Leu Glu Glu		
25	210	215	220

Leu Asp Trp Cys Leu Asp Gln Leu Glu Thr Leu Gln Thr Arg His Ser
225 230 235 240

Val Ser Glu Met Ala Ser Asn Lys Phe Lys Arg Met Leu Asn Arg Glu
245 250 255

5 Leu Thr His Leu Ser Glu Met Ser Arg Ser Gly Asn Gln Val Ser Glu
260 265 270

Phe Ile Ser Asn Thr Phe Leu Asp Lys Gln His Glu Val Glu Ile Pro
275 280 285

Ser Pro Thr Gln Lys Glu Lys Glu Lys Lys Lys Arg Pro Met Ser Gln
10 290 295 300

Ile Ser Gly Val Lys Lys Leu Met His Ser Ser Ser Leu Thr Asn Ser
305 310 315 320

Ser Ile Pro Arg Phe Gly Val Lys Thr Glu Gln Glu Asp Val Leu Ala
325 330 335

15 Lys Glu Leu Glu Asp Val Asn Lys Trp Gly Leu His Val Phe Arg Ile
340 345 350

Ala Glu Leu Ser Gly Asn Arg Pro Leu Thr Val Ile Met His Thr Ile
355 360 365

Phe Gln Glu Arg Asp Leu Leu Lys Thr Phe Lys Ile Pro Val Asp Thr
20 370 375 380

Leu Ile Thr Tyr Leu Met Thr Leu Glu Asp His Tyr His Ala Asp Val
385 390 395 400

Ala Tyr His Asn Asn Ile His Ala Ala Asp Val Val Gln Ser Thr His
405 410 415

25 Val Leu Leu Ser Thr Pro Ala Leu Glu Ala Val Phe Thr Asp Leu Glu

	420	425	430													
	Ile	Leu	Ala	Ala	Ile	Phe	Ala	Ser	Ala	Ile	His	Asp	Val	Asp	His	Pro
	435	440	445													
	Gly	Val	Ser	Asn	Gln	Phe	Leu	Ile	Asn	Thr	Asn	Ser	Glu	Leu	Ala	Leu
5	450	455	460													
	Met	Tyr	Asn	Asp	Ser	Ser	Val	Leu	Glu	Asn	His	His	Leu	Ala	Val	Gly
	465	470	475	480												
	Phe	Lys	Leu	Leu	Gln	Glu	Glu	Asn	Cys	Asp	Ile	Phe	Gln	Asn	Leu	Thr
	485	490	495													
10	Lys	Lys	Gln	Arg	Gln	Ser	Leu	Arg	Lys	Met	Val	Ile	Asp	Ile	Val	Leu
	500	505	510													
	Ala	Thr	Asp	Met	Ser	Lys	His	Met	Asn	Leu	Leu	Ala	Asp	Leu	Lys	Thr
	515	520	525													
	Met	Val	Glu	Thr	Lys	Lys	Val	Thr	Ser	Ser	Gly	Val	Leu	Leu	Leu	Asp
15	530	535	540													
	Asn	Tyr	Ser	Asp	Arg	Ile	Gln	Val	Leu	Gln	Asn	Met	Val	His	Cys	Ala
	545	550	555	560												
	Asp	Leu	Ser	Asn	Pro	Thr	Lys	Pro	Leu	Gln	Leu	Tyr	Arg	Gln	Trp	Thr
	565	570	575													
20	Asp	Arg	Ile	Met	Glu	Glu	Phe	Phe	Arg	Gln	Gly	Asp	Arg	Glu	Arg	Glu
	580	585	590													
	Arg	Gly	Met	Glu	Ile	Ser	Pro	Met	Cys	Asp	Lys	His	Asn	Ala	Ser	Val
	595	600	605													
	Glu	Lys	Ser	Gln	Val	Gly	Phe	Ile	Asp	Tyr	Ile	Val	His	Pro	Leu	Trp
25	610	615	620													

Glu Thr Trp Ala Asp Leu Val His Pro Asp Ala Gln Asp Ile Leu Asp
625 630 635 640
Thr Leu Glu Asp Asn Arg Glu Trp Tyr Gln Ser Thr Ile Pro Gln Ser
645 650 655
5 Pro Ser Pro Ala Pro Asp Asp Pro Glu Glu Gly Arg Gln Gly Gln Thr
660 665 670
Glu Lys Phe Gln Phe Glu Leu Thr Leu Glu Glu Asp Gly Glu Ser Asp
675 680 685
Thr Glu Lys Asp Ser Gly Ser Gln Val Glu Glu Asp Thr Ser Cys Ser
10 690 695 700
Asp Ser Lys Thr Leu Cys Thr Gln Asp Ser Glu Ser Thr Glu Ile Pro
705 710 715 720
Leu Asp Glu Gln Val Glu Glu Glu Ala Val Gly Glu Glu Glu Glu Ser
725 730 735
15 Gln Pro Glu Ala Cys Val Ile Asp Asp Arg Ser Pro Asp Thr
740 745 750

<210> 6
<211> 679
20 <212> PRT
<213> Homo sapiens
<220>
<221> PDE4D8
<222> (1) .. (679)
25 <223>

<220>

<221> isoform_specific_N-terminus

<222> (1)..(23)

<223>

5 <220>

<221> LF1_splice_site

<222> (23)..(23)

<223>

<220>

10 <221> UCR1_start

<222> (56)..(56)

<223>

<220>

<221> UCR1_start-13

15 <222> (43)..(43)

<223>

<220>

<221> Ser54

<222> (60)..(60)

20 <223>

<220>

<221> Ser579

<222> (585)..(585)

<223>

25 <400> 6

Met Ser Ile Ile Met Lys Pro Arg Ser Arg Ser Thr Ser Ser Leu Arg
1 5 10 15
Thr Ala Glu Ala Lys Leu Phe Asp Val Asp Asn Gly Thr Ser Ala Gly
 20 25 30
5 Arg Ser Pro Leu Asp Pro Met Thr Ser Pro Gly Ser Gly Leu Ile Leu
 35 40 45
Gln Ala Asn Phe Val His Ser Gln Arg Arg Glu Ser Phe Leu Tyr Arg
 50 55 60
Ser Asp Ser Asp Tyr Asp Leu Ser Pro Lys Ser Met Ser Arg Asn Ser
10 65 70 75 80
Ser Ile Ala Ser Asp Ile His Gly Asp Asp Leu Ile Val Thr Pro Phe
 85 90 95
Ala Gln Val Leu Ala Ser Leu Arg Thr Val Arg Asn Asn Phe Ala Ala
 100 105 110
15 Leu Thr Asn Leu Gln Asp Arg Ala Pro Ser Lys Arg Ser Pro Met Cys
 115 120 125
Asn Gln Pro Ser Ile Asn Lys Ala Thr Ile Thr Glu Glu Ala Tyr Gln
 130 135 140
Lys Leu Ala Ser Glu Thr Leu Glu Glu Leu Asp Trp Cys Leu Asp Gln
20 145 150 155 160
Leu Glu Thr Leu Gln Thr Arg His Ser Val Ser Glu Met Ala Ser Asn
 165 170 175
Lys Phe Lys Arg Met Leu Asn Arg Glu Leu Thr His Leu Ser Glu Met
 180 185 190
25 Ser Arg Ser Gly Asn Gln Val Ser Glu Phe Ile Ser Asn Thr Phe Leu

	195		200		205
	Asp Lys Gln His Glu Val Glu Ile Pro Ser Pro Thr Gln Lys Glu Lys				
	210		215		220
	Glu Lys Lys Lys Arg Pro Met Ser Gln Ile Ser Gly Val Lys Lys Leu				
5	225		230		235 240
	Met His Ser Ser Ser Leu Thr Asn Ser Ser Ile Pro Arg Phe Gly Val				
		245		250	255
	Lys Thr Glu Gln Glu Asp Val Leu Ala Lys Glu Leu Glu Asp Val Asn				
	260		265		270
10	Lys Trp Gly Leu His Val Phe Arg Ile Ala Glu Leu Ser Gly Asn Arg				
	275		280		285
	Pro Leu Thr Val Ile Met His Thr Ile Phe Gln Glu Arg Asp Leu Leu				
	290		295		300
	Lys Thr Phe Lys Ile Pro Val Asp Thr Leu Ile Thr Tyr Leu Met Thr				
15	305		310		315 320
	Leu Glu Asp His Tyr His Ala Asp Val Ala Tyr His Asn Asn Ile His				
		325		330	335
	Ala Ala Asp Val Val Gln Ser Thr His Val Leu Leu Ser Thr Pro Ala				
	340		345		350
20	Leu Glu Ala Val Phe Thr Asp Leu Glu Ile Leu Ala Ala Ile Phe Ala				
	355		360		365
	Ser Ala Ile His Asp Val Asp His Pro Gly Val Ser Asn Gln Phe Leu				
	370		375		380
	Ile Asn Thr Asn Ser Glu Leu Ala Leu Met Tyr Asn Asp Ser Ser Val				
25	385		390		395 400

Leu Glu Asn His His Leu Ala Val Gly Phe Lys Leu Leu Gln Glu Glu
405 410 415
Asn Cys Asp Ile Phe Gln Asn Leu Thr Lys Lys Gln Arg Gln Ser Leu
420 425 430
5 Arg Lys Met Val Ile Asp Ile Val Leu Ala Thr Asp Met Ser Lys His
435 440 445
Met Asn Leu Leu Ala Asp Leu Lys Thr Met Val Glu Thr Lys Lys Val
450 455 460
Thr Ser Ser Gly Val Leu Leu Leu Asp Asn Tyr Ser Asp Arg Ile Gln
10 465 470 475 480
Val Leu Gln Asn Met Val His Cys Ala Asp Leu Ser Asn Pro Thr Lys
485 490 495
Pro Leu Gln Leu Tyr Arg Gln Trp Thr Asp Arg Ile Met Glu Glu Phe
500 505 510
15 Phe Arg Gln Gly Asp Arg Glu Arg Glu Arg Gly Met Glu Ile Ser Pro
515 520 525
Met Cys Asp Lys His Asn Ala Ser Val Glu Lys Ser Gln Val Gly Phe
530 535 540
Ile Asp Tyr Ile Val His Pro Leu Trp Glu Thr Trp Ala Asp Leu Val
20 545 550 555 560
His Pro Asp Ala Gln Asp Ile Leu Asp Thr Leu Glu Asp Asn Arg Glu
565 570 575
Trp Tyr Gln Ser Thr Ile Pro Gln Ser Pro Ser Pro Ala Pro Asp Asp
580 585 590
25 Pro Glu Glu Gly Arg Gln Gly Gln Thr Glu Lys Phe Gln Phe Glu Leu

595 600 605
Thr Leu Glu Glu Asp Gly Glu Ser Asp Thr Glu Lys Asp Ser Gly Ser
610 615 620
Gln Val Glu Glu Asp Thr Ser Cys Ser Asp Ser Lys Thr Leu Cys Thr
5 625 630 635 640
Gln Asp Ser Glu Ser Thr Glu Ile Pro Leu Asp Glu Gln Val Glu Glu
645 650 655
Glu Ala Val Gly Glu Glu Glu Glu Ser Gln Pro Glu Ala Cys Val Ile
660 665 670
10 Asp Asp Arg Ser Pro Asp Thr
675

<210> 7
<211> 809
15 <212> PRT
<213> Homo sapiens
<220>
<221> PDE4D4
<222> (1)..(809)
20 <223>
<220>
<221> LF1_splice_site
<222> (153)..(153)
<223>
25 <220>

<221> UCR_1_start
<222> (186)..(186)
<223>
<220>
5 <221> UCR1_start_-13
<222> (173)..(173)
<223>
<220>
<221> Ser54
10 <222> (190)..(190)
<223>
<220>
<221> Ser579
<222> (715)..(715)
15 <223>
<400> 7

Met Glu Ala Glu Gly Ser Ser Ala Pro Ala Arg Ala Gly Ser Gly Glu
1 5 10 15
Gly Ser Asp Ser Ala Gly Gly Ala Thr Leu Lys Ala Pro Lys His Leu
20 20 25 30
Trp Arg His Glu Gln His His Gln Tyr Pro Leu Arg Gln Pro Gln Phe
35 40 45
Arg Leu Leu His Pro His His His Leu Pro Pro Pro Pro Pro Pro Ser
50 55 60
25 Pro Gln Pro Gln Pro Gln Cys Pro Leu Gln Pro Pro Pro Pro Pro Pro

	65	70	75	80												
	Leu	Pro	Pro	Pro	Pro	Pro	Pro	Gly	Ala	Ala	Arg	Gly	Arg	Tyr	Ala	
				85				90						95		
	Ser	Ser	Gly	Ala	Thr	Gly	Arg	Val	Arg	His	Arg	Gly	Tyr	Ser	Asp	Thr
5				100				105						110		
	Glu	Arg	Tyr	Leu	Tyr	Cys	Arg	Ala	Met	Asp	Arg	Thr	Ser	Tyr	Ala	Val
				115				120						125		
	Glu	Thr	Gly	His	Arg	Pro	Gly	Leu	Lys	Lys	Ser	Arg	Met	Ser	Trp	Pro
				130				135						140		
10	Ser	Ser	Phe	Gln	Gly	Leu	Arg	Arg	Phe	Asp	Val	Asp	Asn	Gly	Thr	Ser
				145				150						155		160
	Ala	Gly	Arg	Ser	Pro	Leu	Asp	Pro	Met	Thr	Ser	Pro	Gly	Ser	Gly	Leu
					165					170					175	
	Ile	Leu	Gln	Ala	Asn	Phe	Val	His	Ser	Gln	Arg	Arg	Glu	Ser	Phe	Leu
15				180					185					190		
	Tyr	Arg	Ser	Asp	Ser	Asp	Tyr	Asp	Leu	Ser	Pro	Lys	Ser	Met	Ser	Arg
				195					200					205		
	Asn	Ser	Ser	Ile	Ala	Ser	Asp	Ile	His	Gly	Asp	Asp	Leu	Ile	Val	Thr
				210					215					220		
20	Pro	Phe	Ala	Gln	Val	Leu	Ala	Ser	Leu	Arg	Thr	Val	Arg	Asn	Asn	Phe
				225					230					235		240
	Ala	Ala	Leu	Thr	Asn	Leu	Gln	Asp	Arg	Ala	Pro	Ser	Lys	Arg	Ser	Pro
					245					250					255	
	Met	Cys	Asn	Gln	Pro	Ser	Ile	Asn	Lys	Ala	Thr	Ile	Thr	Glu	Glu	Ala
25				260						265					270	

	Tyr	Gln	Lys	Leu	Ala	Ser	Glu	Thr	Leu	Glu	Glu	Leu	Asp	Trp	Cys	Leu
	275						280						285			
	Asp	Gln	Leu	Glu	Thr	Leu	Gln	Thr	Arg	His	Ser	Val	Ser	Glu	Met	Ala
	290						295						300			
5	Ser	Asn	Lys	Phe	Lys	Arg	Met	Leu	Asn	Arg	Glu	Leu	Thr	His	Leu	Ser
	305						310					315			320	
	Glu	Met	Ser	Arg	Ser	Gly	Asn	Gln	Val	Ser	Glu	Phe	Ile	Ser	Asn	Thr
							325					330			335	
	Phe	Leu	Asp	Lys	Gln	His	Glu	Val	Glu	Ile	Pro	Ser	Pro	Thr	Gln	Lys
10			340						345					350		
	Glu	Lys	Glu	Lys	Lys	Lys	Arg	Pro	Met	Ser	Gln	Ile	Ser	Gly	Val	Lys
			355						360					365		
	Lys	Leu	Met	His	Ser	Ser	Ser	Leu	Thr	Asn	Ser	Ser	Ile	Pro	Arg	Phe
			370						375					380		
15	Gly	Val	Lys	Thr	Glu	Gln	Glu	Asp	Val	Leu	Ala	Lys	Glu	Leu	Glu	Asp
			385						390				395		400	
	Val	Asn	Lys	Trp	Gly	Leu	His	Val	Phe	Arg	Ile	Ala	Glu	Leu	Ser	Gly
									405					410		415
	Asn	Arg	Pro	Leu	Thr	Val	Ile	Met	His	Thr	Ile	Phe	Gln	Glu	Arg	Asp
20			420							425				430		
	Leu	Leu	Lys	Thr	Phe	Lys	Ile	Pro	Val	Asp	Thr	Leu	Ile	Thr	Tyr	Leu
			435							440				445		
	Met	Thr	Leu	Glu	Asp	His	Tyr	His	Ala	Asp	Val	Ala	Tyr	His	Asn	Asn
			450							455				460		
25	Ile	His	Ala	Ala	Asp	Val	Val	Gln	Ser	Thr	His	Val	Leu	Leu	Ser	Thr

465	470	475	480
Pro Ala Leu Glu Ala Val Phe Thr Asp Leu Glu Ile Leu Ala Ala Ile			
	485	490	495
Phe Ala Ser Ala Ile His Asp Val Asp His Pro Gly Val Ser Asn Gln			
5	500	505	510
Phe Leu Ile Asn Thr Asn Ser Glu Leu Ala Leu Met Tyr Asn Asp Ser			
	515	520	525
Ser Val Leu Glu Asn His His Leu Ala Val Gly Phe Lys Leu Leu Gln			
	530	535	540
10	Glu Glu Asn Cys Asp Ile Phe Gln Asn Leu Thr Lys Lys Gln Arg Gln		
	545	550	555
	Ser Leu Arg Lys Met Val Ile Asp Ile Val Leu Ala Thr Asp Met Ser		
	565	570	575
Lys His Met Asn Leu Leu Ala Asp Leu Lys Thr Met Val Glu Thr Lys			
15	580	585	590
Lys Val Thr Ser Ser Gly Val Leu Leu Leu Asp Asn Tyr Ser Asp Arg			
	595	600	605
Ile Gln Val Leu Gln Asn Met Val His Cys Ala Asp Leu Ser Asn Pro			
	610	615	620
20	Thr Lys Pro Leu Gln Leu Tyr Arg Gln Trp Thr Asp Arg Ile Met Glu		
	625	630	635
	Glu Phe Phe Arg Gln Gly Asp Arg Glu Arg Glu Arg Gly Met Glu Ile		
	645	650	655
Ser Pro Met Cys Asp Lys His Asn Ala Ser Val Glu Lys Ser Gln Val			
25	660	665	670

Gly Phe Ile Asp Tyr Ile Val His Pro Leu Trp Glu Thr Trp Ala Asp
 675 680 685
 Leu Val His Pro Asp Ala Gln Asp Ile Leu Asp Thr Leu Glu Asp Asn
 690 695 700
 5 Arg Glu Trp Tyr Gln Ser Thr Ile Pro Gln Ser Pro Ser Pro Ala Pro
 705 710 715 720
 Asp Asp Pro Glu Glu Gly Arg Gln Gly Gln Thr Glu Lys Phe Gln Phe
 725 730 735
 Glu Leu Thr Leu Glu Glu Asp Gly Glu Ser Asp Thr Glu Lys Asp Ser
 10 740 745 750
 Gly Ser Gln Val Glu Glu Asp Thr Ser Cys Ser Asp Ser Lys Thr Leu
 755 760 765
 Cys Thr Gln Asp Ser Glu Ser Thr Glu Ile Pro Leu Asp Glu Gln Val
 770 775 780
 15 Glu Glu Glu Ala Val Gly Glu Glu Glu Glu Ser Gln Pro Glu Ala Cys
 785 790 795 800
 Val Ile Asp Asp Arg Ser Pro Asp Thr
 805

20 <210> 8

<211> 1974

<212> DNA

<213> Homo sapiens

<220>

25 <221> PDE4D_core_construct

<222> (1) .. (1974)

<223>

<400> 8

	ttt gatgtgg acaatggcac atctgcggga cggagtcctt tggatcccat gaccagccca	60
5	ggatccgggc taattctcca agcaaatttt gtccacagtc aacgacggga gtccttcttg	120
	tatcgatccg acagcgatta tgacctctct ccaaagtcta tgtcccggaa ctctccatt	180
	gccagt gata tacacggaga tgacttgatt gtgactccat ttgctcaggt cttggccagt	240
	ctgcgaactg tacgaaacaa ctttgctgca ttaactaatt tgcaagatcg agcacctagc	300
	aaaagatcac ccatgtgcaa ccaaccatcc atcaacaaag ccaccataac agaggaggcc	360
10	taccagaaac tggccagcga gaccctggag gagctggact ggtgtctgga ccagctagag	420
	accctacaga ccaggcactc cgtcagtga atggcctcca acaagttaa aaggatgctt	480
	aatcgggagc tcacccatct ctctgaaatg agtcgggtctg gaaatcaagt gtcagagttt	540
	atatcaaaca cattcttaga taagcaacat gaagtggaaa ttccttctcc aactcagaag	600
	gaaaaggaga aaaagaaaag accaatgtct cagatcagtg gagtcaagaa attgatgcac	660
15	agctctagtc tgactaattc aagtatccca aggtttggag ttaaaactga acaagaagat	720
	gtccttgcca aggaactaga agatgtgaac aaatgggggtc ttcattgtttt cagaatagca	780
	gagttgtctg gtaaccggcc cttgactgtt atcatgcaca ccatttttca ggaacgggat	840
	ttattaaaaa catttaaaat tccagtagat actttaatta catatcttat gactctcgaa	900
	gaccattacc atgctgatgt ggcctatcac aacaatatcc atgctgcaga tgttgtccag	960
20	tctactcatg tgctattatc tacacctgct ttggaggctg tgtttacaga tttggagatt	1020
	cttgcagcaa tttttgccag tgcaatacat gatgtagatc atcctgggtg gtccaatcaa	1080
	tttctgatca atacaaactc tgaacttgcc ttgatgtaca atgattcctc agtcttagag	1140
	aaccatcatt tggctgtggg ctttaaattg cttcaggaag aaaactgtga cattttccag	1200
	aatttgacca aaaaacaaag acaatcttta aggaaaatgg tcattgacat cgtacttgca	1260
25	acagatatgt caaaacacat gaatctactg gctgatttga agactatggt tgaaactaag	1320

	aaagtgacaa gctctggagt tcttcttctt gataattatt ccgataggat tcaggttctt	1380
	cagaatatgg tgcactgtgc agatctgagc aaccaacaa agcctctcca gctgtaccgc	1440
	cagtggacgg accggataat ggaggagttc ttccgccaag gagaccgaga gagggaacgt	1500
	ggcatggaga taagcccat gtgtgacaag cacaatgctt ccgtggaaaa atcacaggtg	1560
5	ggcttcatag actatattgt tcatccctc tgggagacat gggcagacct cgtccacct	1620
	gacgcccagg atattttgga cactttggag gacaatcgtg aatggtacca gagcacaatc	1680
	cctcagagcc cctctcctgc acctgatgac ccagaggagg gccggcaggg tcaaactgag	1740
	aaattccagt ttgaactaac tttagaggaa gatggtgagt cagacacgga aaaggacagt	1800
	ggcagtcaag tggaagaaga cactagctgc agtgactcca agactctttg tactcaagac	1860
10	tcagagtcta ctgaaattcc ccttgatgaa caggttgaag aggaggcagt aggggaagaa	1920
	gaggaaagcc agcctgaagc ctgtgtcata gatgatcgtt ctctgacac gtaa	1974

This Page Blank (uspto)